University of Alberta

The Structural Basis of Ubiquitin Conjugating Enzyme Function

by

Chantelle S. Gwozd ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of
the requirements for the degree of Doctor of Philosophy.

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To Todd and Matthew, who made the time
spent writing this manuscript more enjoyable

ABSTRACT

The ubiquitin system is a highly conserved pathway of protein degradation present in all eukaryotic cells. Ubiquitin conjugating enzymes, or E2s, are a class of proteins that aid in protein ubiquitination. E2s are essential for many cellular processes and potentially interact with a number of different proteins, including substrates and accessory proteins. The structural requirements, however, of specific E2 functions are largely unknown. This manuscript details experiments that were done in an effort to better understand the structural basis of E2 function.

The stress-related E2 UBC4, from *Saccharomyces cerevisiae,* monoubiquitinates itself *in vivo* in an intermolecular reaction. The site of ubiquitination was localized to a single lysine (K144). Substitution of a second lysine within UBC4 (K64) to an arginine resulted in the loss of ubiquitination at K144. Substitution of either K64 or K144 had no effect on the known *in vivo* functions associated with UBC4. Cross-linking analysis demonstrated that UBC4 monomers interact directly and specifically with one another *in vitro*. This, coupled with the *in vivo* observations, support the view that UBC4 monomers form homocomplexes *in vivo.*

To identify determinants of specific *in vivo* functions, substitutions were made in the DNA repair E2 RAD6 in an effort to generate a protein with UBC4 function. Two key residues were identified that conferred significant UBC4 activity to RAD6. This suggests that the selectivity of E2s can be mediated by only a small number of surface residues. As well, amino acid residues were identified that participate in the DNA repair activity of RAD6. An overall picture of the present knowledge of UBC4 and RAD6 specificity regions is also presented.

Finally an effort was made to reproduce the degradation, in yeast, of a specific target of UBC4, p53. p53 has previously been shown to be degraded in a ubiquitin dependent manner in cells infected with human papilloma virus. This reaction is mediated by the human homologue of UBC4. It was found that p53 was stable in yeast even in the

presence of the human papilloma virus protein E6 and the cellular protein E6AP, components necessary for p53 degradation *in vitro*.

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ABBREVIATIONS

Asn	Asparagine
at	*Arabidopsis thaliana*
A. thaliana	*Arabidopsis thaliana*
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BS3	bis(sulfosuccinimidyl) suberate
C	Carboxyl
ce	*Caenorhabditis elegans*
C. elegans	*Caenorhabditis elegans*
cpm	Counts per minute
dm	*Drosophila melanogaster*
D. melanogaster	*Drosophila melanogaster*
DTT	Dithiothreitol
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin protein ligase
E6AP	E6 associated protein
ECL	Enhanced chemiluminescence
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
HA	Hemagglutinin
Hepes	4-(2-hydroxyethyl)-1-piperazine-1-ethane sulfonic acid
HPV	Human papilloma virus
hs	*Homo sapiens*
kDa	kilodalton
Lys	Lysine

mUb	myc-tagged ubiquitin
N	amino
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
Phe	Phenylalanine
PMSF	Phenylmethylsulfonyl fluoride
Pro	Proline
rpm	revolutions per minute
RNA	ribonucleic acid
sc	*Saccharomyces cerevisiae*
S. cerevisiae	*Saccharomyces cerevisiae*
SD	Synthetic defined
SDS	Sodium dodecylsulfate
Ser	Serine
sp	*Schizosaccharomyces pombe*
S. pombe	*Schizosaccharomyces pombe*
TCA	Trichloroacetic acid
Tyr	Tyrosine
Ub	Ubiquitin
UFD	Ubiquitin fusion degradation
Ub-met-β-gal	Ubiquitin met-β-galactosidase
Ub-pro-β-gal	Ubiquitin pro-β-galactosidase
UV	Ultraviolet

Chapter I

General Introduction

All living cells require the ability to adapt to their environment in order to survive. Cells respond to environmental signals by altering the activities of numerous intracellular proteins. This can be achieved by either covalent modification, alteration of the amount, or compartmentalization of a protein, or by protein or allosteric interactions. The concentration of a protein can be adjusted by either changing the protein's rate of synthesis or rate of degradation, with the latter process having the advantage of being fast and irreversible. An important nonlysomsomal pathway of protein degradation in eukaryotic cells is the ubiquitin (Ub) conjugation system.

Ub is a small 76 amino acid protein found in all eukaryotic cells. Ub was first identified as a heat stable molecule that was required for adenosine triphosphate (ATP) dependent protein degradation in rabbit reticulocyte extracts (Ciechanover *et al.*, 1978; Wilkinson *et al.*, 1980). Further analysis revealed a complex cascade of enzymes that catalyze the attachment of Ub to target proteins (Hershko *et al.*, 1983). Ub-protein conjugate formation often leads to degradation of the target protein, but several nonproteolytic functions for Ub have been described (see below).

The first step in protein ubiquitination is the activation of Ub in a reaction involving ATP (figure 1). The carboxyl group of Ub's terminal glycine reacts with the active site cysteine of an enzyme known as the Ub activating enzyme, or E1, to form a thiolester, with the concomitant hydrolysis of ATP. Next, through trans-thiol esterification, Ub is transferred to a cysteine residue in one of a class of proteins known as Ub conjugating enzymes or E2s. Finally, Ub is attached to the ε amino group of a lysine residue within a protein substrate, with or without the aid of a ubiquitin protein ligase or E3. Recent results suggest that E3s, in addition to being involved in substrate selection, may play a more active role in ubiquitination by transferring Ub from E2s to protein substrates through a Ub-E3 thiolester intermediate (Rolfe *et al.*, 1995; Scheffner *et al.*, 1995). Once Ub has been attached to a protein, additional Ub moieties can be added to the first to build a Ub chain which is recognized by the 26S proteasome resulting in the subsequent degradation of the protein. Alternatively, Ub can be removed from the protein via the action of one of a class of deubiquitinating enzymes.

The Ub system targets both damaged proteins as well as short-lived regulatory proteins. It is unclear exactly how proteins are selected by the Ub system, but several cellular targets have been identified. They include transcription factors, protein kinases, cyclins, cell surface receptors, and transport proteins (reviewed in Hochstrasser, 1995). The identification of an ever increasing number of proteins degraded by the Ub system lends support to the idea that this pathway plays a major role in selective cellular proteolysis.

As mentioned previously, Ub has several nonproteolytic functions. A number of stable Ub conjugates have been identified and include histones H2A and H2B, the *Drosophila* actin protein, the platelet derived growth factor receptor, the growth hormone receptor, and the lymphocyte homing receptor (Jentsch, 1992). The stability of these proteins has led to the suggestion that ubiquitination may in some cases serve to modulate protein activity, much in the same way as protein phosphorylation (Jentsch, 1992). In support of this is the recent observation by Chen and colleagues (1996) that activation of the IκBα kinase by Ub conjugate formation does not involve proteolysis. Ubiquitin conjugate formation has also been shown to cause internalization of plasma membrane receptors (Egner and Kuchler, 1996; Hicke and Riezman, 1996) and protein processing (Palombella *et al.*, 1994; Orian *et al.*, 1995). Finally Ub can act as a chaperone when present in a linear fusion with another protein (Finley *et al.*, 1989).

The Ub system is coordinated by the action of numerous proteins, and these are described briefly below. As well, a detailed discussion is provided of two E2s relevant to my thesis work, followed by a summary of what is presently known about E2 structure and function.

Ubiquitin

Ub is the most highly conserved known eukaryotic protein, with only three amino acids different between the human and yeast proteins. Determination of the crystal structure of Ub (Vijay-Kumar *et al.*, 1987) revealed that it is a compact globular protein with a short protruding carboxyl (C) terminus. Ub is encoded by two classes of genes. The first class is constitutively expressed and consists of *Ub* fused to the 5' end of sequences encoding ribosomal proteins (Finley *et al.*, 1989; Redman and Rechsteiner, 1989). The gene products are linear Ub fusions and are processed by cleavage enzymes to produce Ub and the ribosomal proteins. The Ub moieties act as molecular chaperones by facilitating ribosomal assembly (Finley *et al.*, 1989), but may also be required for coordination of protein synthesis and degradation. The second class of Ub genes consists of several *Ub* sequences fused in a head to tail manner. The expressed protein is a linear polyUb fusion that is rapidly processed by cleavage enzymes producing free Ub monomers (Özkaynak *et al.*, 1984; Finley *et al.*, 1987). The final Ub moiety in the polyprotein has an additional amino acid at its C terminus that is removed and is probably required to prevent conjugation of unprocessed polyUb (Özkaynak *et al.*, 1984). The polyUb gene is dispensable for exponential growth in yeast, but is required under stress conditions where its expression is induced (Finley *et al.*, 1987). This allows Ub levels to be increased rapidly when protein degradation is required.

Ubiquitin Activating Enzyme (E1)

The essential first step in Ub conjugate formation is the activation of Ub in a reaction requiring ATP. Ub activating enzyme, or E1, first catalyzes the adenylation of the C terminal glycine of Ub. Ub is then transferred to a cysteine residue within E1 to form a Ub thiolester intermediate. E1 genes have been cloned from a variety of organisms including yeast, plants, and mammals. The E1 gene products range in size from 110-120 kDa and have highly similar sequences. The *Saccharomyces cerevisiae* (*S. cerevisiae*) E1 UBA1 is located in the cytosol and nucleus (Dohmen *et al.*, 1995) and is essential for cell viability.

Recently a second gene with high sequence similarity to UBA1 has been cloned from yeast. This gene, labeled *UBA2*, encodes a 71 kDa protein that is localized to the nucleus and, like UBA1, is also essential for cell viability (Dohmen *et al.*, 1995). UBA2 contains a cysteine residue within an E1 active site consensus sequence, but, under reaction conditions appropriate for UBA1, UBA2 does not form a Ub thiolester conjugate. The cysteine residue, however, is required for an essential function of UBA2 because mutation of this position results in a protein that is unable to support growth under conditions that repress expression of the chromosomal copy of *UBA2*. Because UBA2 activity absolutely requires the conserved cysteine residue, but a Ub thiolester conjugate cannot be detected, it has been speculated that UBA2 may instead be an E2 (Dohmen *et al.*, 1995). If true this would be the first instance of an E2 with a protein sequence that resembles E1 but not other E2s.

Ubiquitin Conjugating Enzymes (E2)

The next step in the Ub pathway is the transfer of Ub from E1 to the active site cysteine of a Ub conjugating enzyme or E2. Thirteen genes encoding E2 proteins have been isolated from *S. cerevisiae* (UBC1-10 are depicted in figure 2). Deletion of these genes in yeast results in distinct phenotypes and has implicated E2s in a variety of cellular processes including DNA repair (Jentsch *et al.*, 1987), cell cycle progression (Goebl *et al.*, 1988; Seufert *et al.*, 1995), response to environmental stress (Seufert and Jentsch, 1990), sporulation (Jentsch *et al.*, 1987; Jentsch, 1992), cadmium tolerance (Jungmann *et al.*, 1993), and peroxisome biogenesis (Wiebel *et al.*, 1992). From the wide range of phenotypes displayed by E2 null mutants, it is clear E2s must target, directly or indirectly, different proteins.

All E2s possess a catalytic domain of approximately 150 amino acids with at least 25% sequence identity. Within this catalytic domain is a conserved active site cysteine necessary for thiolester formation with Ub. Four classes of E2s have been described based on their primary structures. Class I enzymes consist of only the catalytic domain while the other three classes contain this domain as well as additional sequences. Class II enzymes contain C terminal extensions or tails that are unrelated with respect to their amino acid sequences, but are often highly charged. The tail domains are required for a variety of functions including target recognition (Jentsch *et al.*, 1987; Sung *et al.*, 1988; Sullivan and Vierstra, 1991), E3 recognition (Dohmen *et al.*, 1991), E2 dimerization (Ptak *et al.*, 1994; Silver *et al.*, 1992), autoregulation (Banerjee *et al.*, 1993), membrane anchoring (Sommer and Jentsch, 1993), or formation of alternative Ub chains (Hodgins *et al.*, 1996). Tail domain function will be discussed in some detail later. Four class III E2s have recently been identified. These enzymes have amino (N) terminal extensions but no tail domains (Matuschewski *et al.*, 1996; Nuber *et al.*, 1996). The significance of the N terminal extensions is unclear, but because of their hypervariability they are thought to be, like the tail domains, involved in specificity or regulation. These activities may be carried out by protein phosphorylation as the N terminal extensions contain putative phosphorylation sites. Finally at least one E2 contains both an N and C terminal extension (Jentsch, 1992).

Some E2s have been shown to interact with each other to form higher order complexes. A number of groups have presented *in vitro* chromatographic evidence that E2s can form stable dimeric or multimeric complexes (Pickart and Rose, 1985; Haas and Bright, 1988; Girod and Vierstra, 1993). Cross linking analysis has been used to show that the E2s CDC34 and UBC4 form homodimers (Ptak *et al.*, 1994; Gwozd *et al.*, 1995). Genetic evidence also exists for the association of E2s. A CDC34 derivative consisting of only the catalytic domain (CDC34Δ) can partially complement a temperature sensitive but not a null *cdc34* strain (Silver *et al.*, 1992). The simplest explanation is that CDC34Δ associates with the temperature sensitive cdc34 protein to form a functional complex, but CDC34Δ alone (i.e., in the null strain) is not active. These results suggest that CDC34 monomers can associate. Finally Chen and colleagues (1993) have shown, using the yeast two hybrid system, that UBC7 interacts with both itself and UBC6. Both UBC6 and UBC7 are involved in the degradation of the transcription regulator MATα2 and the two hybrid results suggest that heterodimer formation is required for this activity. The consequence of E2 dimerization is still unclear, but could be used to increase the number of substrate recognition sites through combinatorial association of E2s.

Ubiquitin Protein Ligases (E3)

In some cases additional proteins are required for the ubiquitination of target proteins, and these have been designated Ub protein ligases or E3s. The first E3 activity was isolated from a rabbit reticulocyte lysate (Hershko *et al.*, 1983) and actually contained two proteins, E3α and E3β (Heller and Hershko, 1990; Reiss and Hershko, 1990). Both of these enzymes are involved in the degradation of proteins based on the identity of the N terminal residue of the substrate. This is known as the N end rule pathway of degradation. E3α binds proteins with N terminal residues that are either basic or bulky hydrophobic, while E3β recognizes small and uncharged N terminal residues. A yeast mutant, in which the gene for the E3α homologue *UBR1* has been knocked out, does not degrade N end rule substrates (Bartel *et al.*, 1990). However, *ubr1* null mutants are only partially defective in sporulation and growth; this lack of a significant phenotype argues that the degradation of proteins based on their N terminal residues is not a major pathway of cellular proteolysis.

An unusual example of an E3 is displayed by the combination of a viral protein, E6, and a cellular protein. Normally p53 steady state levels are very low in cells infected with the human papilloma virus (HPV). It has been demonstrated that the HPV protein E6 stimulates p53 degradation, and this occurs by the Ub pathway. An additional cellular protein is required for degradation and this protein, which binds to E6, is known as E6 associated protein (E6AP). Thus, the E6-E6AP complex functions as an E3 and represents an interesting example of how a virus can redirect Ub mediated protein degradation to its advantage.

In the absence of HPV infection E6AP likely functions as an E3 and targets certain cellular proteins for degradation. Further, a data base search has revealed a number of proteins that contain sequence similarities to E6AP especially in their C terminal 350 residues. These proteins have been designated hect domain proteins (homologous to the E6AP carboxyl terminus, Huibregtse *et al.*, 1995). One of these proteins, RSP5, has been implicated in the degradation of the yeast general amino acid permease and the uracil permease (Hein *et al.*, 1995; Galan *et al.*, 1996). It is likely that other hect domain proteins will be found to function as E3s.

Several E3s have been isolated based on their ability to promote Ub conjugation and degradation of specific proteins. From *S. cerevisiae* one E3 has been partially purified that promotes the monoubiquitination of calmodulin (Parag *et al.*, 1993). Another E3 activity, E3L, has been purified from mammalian tissue and recognizes non N end rule substrates (Gonen *et al.*, 1996). This E3 can target actin, troponin T, and myoD for Ub conjugation and degradation, leading to the suggestion that it may be involved in the degradation of muscle proteins. Recently several E3s involved in cyclin degradation have also been identified (Hershko *et al.*, 1994; King *et al.*, 1995; Sudakin *et al.*, 1995; Willems *et al.*, 1996; Zachariae *et al.*, 1996). Finally, an E3 has been isolated that promotes the rapid processing of the 105 kDa NF-κB precursor protein to the mature p50 subunit (Orian *et al.*, 1995). E3 activities are being isolated at an increasing rate, and it is probable that these few examples are only a small portion of the existing E3s.

Previously it was thought that E3s acted as adapter molecules that brought E2s and target proteins together, and that E2s transferred Ub directly to the target proteins. This view has been challenged recently by the discovery that an E3, E6AP, forms a thiolester with Ub and can donate Ub directly to substrate proteins (Rolfe *et al.*, 1995; Scheffner *et al.*, 1995). Further support for direct E3 transfer of Ub comes from the discovery that other E3s, UBR1 and RSP5, also form a thiolester bond with Ub (Hochstrasser, 1995; Huibregtse *et al.*, 1995). In addition, E3L requires an thiol group for its activity (Gonen *et al.*, 1996) suggesting that it might also function through an essential cysteine residue that binds Ub. Several E3s have been shown to contain binding sites for E2s and target proteins (Hershko et al, 1986; Reiss *et al.*, 1989; Heller and Hershko, 1990; Reiss and Hershko, 1990; Dohmen *et al.*, 1991; Huibregtse *et al.*, 1991; Berleth *et al.*, 1992; Madura *et al.*, 1993). It remains to be determined whether E2s simply charge E3s with Ub or actually participate in substrate selection. If E2s do interact with target proteins, in conjunction with E3s, then different combinations of E2s and E3s would result in a large expansion of the number of substrate binding sites. Some support for the combinatorial interactions of E2s and E3s has come from evidence that an E2, RAD6, can mediate the ubiquitination of proteins with two different E3s (Dohmen *et al.*, 1991; Sung *et al.*, 1991a; Raboy and Kulka, 1994). Another report demonstrates that two different E2s can interact with E6AP (Nuber *et al.*, 1996). UbcH5 and UbcH7 were shown to mediate E6AP dependent ubiquitination of the same substrates *in vitro*, but whether the different E2-E3 combinations actually target separate substrates *in vivo* remains to be demonstrated. Considering that *in vitro* some E2s do not require E3s for Ub conjugation, the role of individual E2s and E3s in target selection and Ub transfer remains to be clarified.

Deubiquitinating Enzymes

In addition to enzymes that conjugate Ub to proteins, enzymes also exist that reverse this reaction. These enzymes are required for release of Ub from proteolytically cleaved substrates, disassembly of polyUb chains, cleavage of Ub fusion proteins, and release of Ub from small amines and thiols. Cleavage by these enzymes occurs at Gly76 of Ub and generates intact Ub which is recycled (Wilkinson, 1995).

Two types of deubiquitinating enzymes exist, Ub carboxyl-terminal hydrolases and Ub specific proteases. Ub carboxyl-terminal hydrolases are thiol proteases of approximately

25 kDa and are responsible for cleaving small esters and amides (such as peptides or the intracellular nucleophile glutathione) from the C terminus of Ub (Mayer and Wilkinson, 1989; Wilkinson *et al.*, 1989; Zhang *et al.*, 1993). Ub specific proteases are also thiol proteases, but are larger, ranging from 100 to 150 kDa. Three of these proteins, UBP1-3 (Baker *et al.*, 1992; Tobias and Varshavsky, 1991), hydrolyze large Ub protein fusions while a fourth enzyme, DOA4 (Papa and Hochstrasser, 1993), is presumably associated with the 26S proteasome (discussed in the next section) and is responsible for releasing Ub chains from the remnants of degraded proteins. Another enzyme, Isopeptidase T, can disassemble unanchored Ub chains (Hadari *et al.*, 1992) thereby increasing the intracellular levels of Ub to prevent inhibitory effects caused by accumulation of Ub chains.

Deubiquitinating enzymes, in addition to the functions described above, have been suggested to serve other roles in the cell. Deubiquitinating enzymes could provide a proofreading function by removing Ub from mistakenly tagged proteins (Rose, 1988). Another hypothesis is that protein turnover rates can be adjusted by altering the activity of Ub specific proteases (Hochstrasser, 1995). Removal of Ub moieties from a substrate would reduce the likelihood of the protein being recognized by the proteasome and degraded. Deubiquitinating enzymes would have to possess some specificity and this would make necessary the presence of numerous Ub specific proteases. In fact a large number, at least fifteen, have been isolated from yeast (Hochstrasser, 1995). Finally, in addition to targeting proteins for proteolysis, ubiquitination has been speculated to modify the activity of proteins in much the same way as protein phosphorylation (Jentsch, 1992). Similar to the action of protein kinases and phosphatases, Ub conjugating and deubiquitinating enzymes could work together to modify protein activity.

The Proteasome

Degradation of proteins targeted for destruction by the Ub pathway occurs through the action of a large cylindrical multisubunit complex found in both the nucleus and cytosol, known as the 26S proteasome. In an energy requiring reaction, the proteasome degrades ubiquitinated proteins to small peptides and free and reutilizable Ub. The proteolytically active core has a sedimentation coefficient of 20S and is composed of four rings, a pair of dimers, with each ring containing seven polypeptides (Peters, 1994; Hilt and Wolf, 1996). All eukaryotic 20S proteasomes are related in terms of function, the amino acid sequences of their subunits, and structure as determined by electron microscopy. The 20S proteasome contains several peptidohydrolase activities including chymotrypsin-like, trypsin-like, and a peptidylglutamyl-hydrolyzing activity (Orlowski *et al.*, 1993). The proteasome is essential for cell viability as deletion in yeast of all but one of the genes encoding these polypeptides is lethal (Heinemeyer et al, 1991; Hilt and Wolf, 1995). Both the archaebacterium *Thermoplasma acidophilum* (Löwe *et al.*, 1995) and the *S. cerevisiae* (Groll *et al.*, 1997) 20S proteasome crystal structures have recently been solved. Approximately 16 different proteins combine to form 19S regulatory complexes (Jentsch and Schlenker, 1995) that, in the presence of ATP, bind to the ends of the 20S proteasome to form the 26S proteasome. These caps are probably needed for recognition of the Ub conjugates and unfolding of the proteolytic substrates (Hilt and Wolf, 1996).

A model of how proteins are degraded by the proteasome has been proposed (Hochstrasser, 1995; Jentsch and Schlenker, 1995). First the multiUb chains of targeted substrates bind to subunit five, one of the 19S cap proteins of the proteasome (Deveraux *et al.*, 1994). Next, tethered proteins are unfolded and threaded into the central cavity of the proteasome where they are cleaved into small peptides. At some point the multiUb chains are freed from the substrate and cleaved into monomers by the action of isopeptidase T. The stable structure of Ub may explain how it avoids being unfolded and degraded by the proteasome.

In conclusion, through a cascade of enzymes, Ub is transferred to specific protein targets which in most situations results in their subsequent degradation by the proteasome.

Ubiquitin Conjugating Enzyme UBC4

Like other E2s, UBC4 was isolated from *S. cerevisiae* based on its ability to bind to a Ub sepharose column in the presence of E1 (Seufert and Jentsch, 1990). The genes encoding UBC4, and a closely related protein UBC5, were cloned and shown to encode 16 kDa proteins that are 92% identical. *UBC4/5* are heat shock and cadmium inducible, and *ubc4ubc5* null mutants are sensitive to these forms of environmental stress as well as to growth on an amino acid analog such as canavanine. Incorporation of canavanine causes proteins to be misfolded, and the inability of *ubc4ubc5* mutants to grow in its presence implicates UBC4/5 in the degradation of abnormal proteins. *ubc4ubc5* null mutants are also deficient in the degradation of short-lived proteins and grow slowly (Seufert and Jentsch, 1990; Jentsch, 1992).

Numerous homologues of *S. cerevisiae UBC4* have subsequently been identified including human (Jensen *et al.*, 1995; Rolfe *et al.*, 1995; Scheffner *et al.*, 1994), rat (Wing and Jain, 1995; Wing *et al.*, 1996), yeast (Damagnez *et al.*, 1995), plant (Girod *et al.*, 1993; Picton *et al.*, 1993; Woo *et al.*, 1994), fly (Trier *et al.*, 1992), and nematode (Zhen *et al.*, 1993). The identification of these proteins as members of the same family has been largely based on the high amount of sequence identity found among these proteins. Further evidence for the functional equivalence of some of these proteins has come from experiments that demonstrate that the *Drosophila melanogaster* (*D. melanogaster*) and the *Caenorhabditis elegans* (*C. elegans*) proteins can complement the *S. cerevisiae ubc4ubc5* null mutant phenotype. The presence of UBC4 related proteins in numerous organisms suggests that these enzymes play a fundamental role in all eukaryotic cells.

Interestingly, multiple isoforms of *S. cerevisiae* related UBC4 proteins have been detected in higher eukaryotes and plants. In *Arabidopsis thaliana* (*A. thaliana*) 5 isoforms have been reported (Girod *et al.*, 1993) while in humans there are at least three isoforms (Scheffner *et al.*, 1994; Jensen *et al.*, 1995; Rolfe *et al.*, 1995). The human isoforms were expressed in all tissues examined, but the amount of each transcript varied (Jensen *et* *al.*, 1995). Of the three rat isoforms that have been isolated, two are diffusely expressed while a third is testis specific (Wing and Jain, 1995; Wing *et al.*, 1996). The testis specific isoform is developmentally regulated; it is absent in early life and appears during puberty. To date only one *UBC4* homologue has been isolated from *C. elegans*, *UBC-2* (Zhen *et al.*, 1993), and it is essential as shown by antisense RNA expression experiments and genetic analysis (Zhen *et al.*, 1996). UBC-2 is developmentally regulated; in embryos and early larvae it is expressed in all tissues, but in adults it is only found in the nervous system. As multiple UBC4 isoforms have been identified in several eukaryotic species, it is likely that *C. elegans* contains other, yet to be identified, UBC4 homologues that may be more diffusely expressed. One reason for the presence of multiple UBC4 related isoforms in eukaryotes is that they may have redundant roles and are present to ensure the performance of some essential function. Nevertheless, the differential expression of the UBC4 isoforms suggests that they mediate the degradation of different target substrates.

Several E2s from fly, mouse, and humans possess sequences similar to UBC4 in their catalytic domains, but have additional N terminal extensions (Matuschewski *et al.*, 1996). These class III enzymes can partially complement the *S. cerevisiae ubc4ubc5* null mutant phenotypes of slow growth and heat sensitivity. This indicates that UBC4 and these enzymes have overlapping specificities and may target similar substrates. However since the different N terminal extensions have limited sequence similarity, they may be responsible for targeting additional substrates specific to the individual enzymes.

Several targets of UBC4 have been identified in *S. cerevisiae*. UBC4 is one of four E2s responsible for degradation of the transcription regulator MATα2 *in vivo* (Chen *et al.*, 1993). Fructose-1,6-bisphosphatase is targeted for degradation by the Ub system under catabolite repression, and its degradation is dependent on UBC4/5 and a functionally related E2, UBC1 (Schork *et al.*, 1995). Calmodulin is monoubiquitinated *in vitro* in the presence of UBC4 and the putative Ub protein ligase E3-CaM (Parag *et al.*, 1993). UBC4 is also involved in the ubiquitination and degradation of the Ub fusion protein Ub-pro-β-galactosidase (Johnson *et al.*, 1992). This artificial substrate consists of Ub fused to a β-galactosidase derivative containing an N terminal proline residue. The proline residue prevents the removal of Ub from the fusion protein by a deubiquitinating enzyme (Bachmair *et al.*, 1986), which in turn allows the Ub moiety to act as a signal for the multiubiquitination of β-galactosidase (Johnson *et al.*, 1992). This has been labeled the Ub fusion degradation (UFD) pathway (Johnson *et al.*, 1995). The physiological substrates of the UFD pathway are not known, but it has been speculated that they may include proteins with Ub like domains (e.g., Banerji *et al.*, 1990; Linnen et al, 1993; Watkins *et al.*, 1993).

Substrates of UBC4 mediated degradation in higher eukaryotes have also been identified. *In vitro* experiments using *Xenopus* egg extracts have shown that cyclin B is ubiquitinated in the presence of a fraction that immunoreacts with UBC4 antibodies, and *S. cerevisiae* UBC4 can complement this activity (King *et al.*, 1995). Also, the tumor suppressor protein p53 is targeted for degradation in an E3 dependent reaction that is mediated by human UBC4 (Scheffner *et al.*, 1994). The specific degradation of these regulatory proteins implies that UBC4 potentially plays a role in cellular regulation.

Finally, UBC4 is able to catalyze the formation of a novel type of multiUb chain. Initial enzymatic cleavage analysis revealed that multiUb chains consisted of Ub monomers linked through an isopeptide bond between the terminal glycine of one Ub moiety and lysine 48 of another (Chau *et al.*, 1989). Using Ub derivatives, in which the lysine residues were changed to arginines (arginine cannot accept Ub), however, allowed the identification of potential Ub linkage sites at four other lysine residues (Arnason and Ellison, 1994; Spence *et al.*, 1995; Baboshina and Haas, 1996). Arnason and Ellison (1994) found that K29 and K63 linkages are absent in the *ubc4ubc5* null mutant. Further K63 linkages, like UBC4/5, appear to play a role in the stress response. Since proteolysis of target proteins relies primarily on chains formed through K48 linkages (Finley *et al.*, 1994), K29 and K63 linkages, catalyzed by UBC4, are speculated to be used for different cellular functions.

Ubiquitin Conjugating Enzyme RAD6

UBC2 was originally isolated as the major E2 activity responsible for monoubiquitinating histones *in vitro* (Jentsch *et al.*, 1987). Cloning of the gene revealed that UBC2 was identical to RAD6 (Jentsch *et al.*, 1987), a protein previously identified as playing a central role in one of three DNA repair pathways in yeast. Strains harboring mutations in *RAD6* grow slowly (Morrison *et al.*, 1988), are highly sensitive to DNA damaging agents such as ultraviolet light (UV), γ-rays, and alkylating agents (Cox and Parry, 1968; Game and Mortimer, 1974; Prakash, 1974), and are defective in induced mutagenesis normally caused by these agents (Prakash, 1974; Lawrence and Christensen, 1976; McKee and Lawrence, 1979). *rad6* mutants are defective in postreplication repair of UV damaged DNA (Prakash, 1981), meiotic recombination (Montelone *et al.*, 1981), and sporulation (Montelone *et al.*, 1981), and show an increase in base pair transitions, GC-TA transversions (Kang *et al.*, 1992), spontaneous and mitotic recombination (Montelone *et al.*, 1981), and transposition of the yeast Ty element (Picologlou *et al.*, 1990; Kang *et al.*, 1992). The *rad6* null mutant also displays a cell cycle defect at elevated temperatures (Ellison *et al.*, 1991). Thus RAD6 is an extremely pleiotropic enzyme.

RAD6 homologues have been found in plants (Sullivan and Vierstra, 1991), yeast (Reynolds *et al.*, 1990), nematodes (Leggett *et al.*, 1995), rabbit reticulocyte lysate (Wing *et al.*, 1992), flies (Koken *et al.*, 1991a), and humans (Schneider *et al.*, 1990; Koken *et al.*, 1991b). All of these proteins lack the tail domain found in *S. cerevisiae* RAD6, except for the *C. elegans* homologue. Several of these homologues (including *S. pombe*, *D. melanogaster*, *C. elegans*, and human) can complement for the loss of DNA repair and UV mutagenesis in the *S. cerevisiae rad6* null mutant. This suggests that RAD6 function is highly conserved in all eukaryotes.

RAD6 appears to conduct all of its functions through its Ub conjugating activity. A RAD6 derivative in which the conserved active site cysteine responsible for Ub binding is mutated to a serine residue can form a stable ester with Ub, but cannot transfer this Ub to a target protein. rad6 C88S behaves as a *rad6* null mutant with respect to UV sensitivity, UV mutagenesis, and sporulation (Sung *et al.*, 1991b).

One function of RAD6 may be to mediate ubiquitination of chromosomal histones as RAD6 is able to catalyze histone ubiquitination *in vitro* (Jentsch *et al.*, 1987; Sung *et al.*, 1988; Haas *et al.*, 1990). Ub-histone conjugates are stable in eukaryotic cells (Goldknopf and Busch, 1975; 1977); therefore it has been speculated that RAD6 plays a role in chromatin structure alteration (Jentsch *et al.*, 1987). Recently Koken and colleagues (1996) have provided indirect evidence for the involvement of RAD6 in chromosome remodeling. They find that the levels of human RAD6 RNA transcripts and protein increase during spermatogenesis, and this coincides with the removal of histones and their subsequent replacement by protamines.

RAD6 is also involved in the degradation of proteins by the N end rule pathway (Dohmen *et al.*, 1991; Sung *et al.*, 1991a). The N end rule relates the identity of the N terminal residue of a protein to its stability (Bachmair *et al.*, 1986; Gonda *et al.*, 1989). E3α from rabbit reticulocytes and its yeast homologue UBR1 cooperate with RAD6 in this activity (Hershko and Ciechanover, 1992; Bartel *et al.*, 1990). *ubr1* null mutants, however, are viable and do not display a characteristic phenotype; therefore it is unlikely that this pathway plays a major role in protein degradation.

In vivo substrates of RAD6 have remained largely elusive, but it is likely that target proteins include other members of the RAD6 DNA repair pathway. Elimination of specific proteins may facilitate the assembly of complexes such as those involved in the repair process, or the disassembly of complexes such as the replication complex, allowing repair enzymes access to the site of a DNA lesion (Lawrence, 1994).

Structure/Function of Ubiquitin Conjugating Enzymes

As discussed in a previous section, E2s contain a conserved catalytic domain and in some cases additional N or C terminal extensions. The C terminal extensions or tails of various E2s bear little resemblance to each other and are found to have a variety of functions. A number of E2s with acidic tails are able to ubiquitinate basic histones *in vitro,* and the tails are necessary for this function (Goebl *et al.*, 1988; Sung *et al.*, 1988; Sullivan and Vierstra, 1991; Kaiser *et al.*, 1995). It has also been shown that fusion of an acidic tail to an E2 normally unable to ubiquitinate histones creates a hybrid protein that now possesses the ability to conjugate Ub to histones (Sullivan and Vierstra, 1991; Kaiser *et al.*, 1995). Thus tail domains can be involved in target recognition. The functions of several E2 tail domains have been determined. The tail of RAD6 is required, in addition to the ubiquitination of histones (Sung *et al.*, 1988), for sporulation (Morrison *et al.*, 1988), N end rule pathway function (Madura *et al.*, 1993, although Sung *et al.* (1991a) find the tail is not essential for this function), and interaction with the E3 UBR1 (Madura *et al.*, 1993). CDC34 requires its tail for E2 dimerization (Ptak *et al.*, 1994), as well as its cell cycle function (Silver *et al.*, 1992; Kolman *et al.*, 1992; Ptak *et al.*, 1994). CDC34 is ubiquitinated *in vitro* as well as *in vivo,* and this modification is also dependent on the tail (Banerjee *et al.*, 1993; Goebl *et al.*, 1994). *In vitro* ubiquitination occurs through an intramolecular reaction and results in Ub being conjugated to one of four lysines in the CDC34 tail (Banerjee *et al.*, 1993). Autoubiquitination gives a hint as to the spatial relationship between the catalytic and tail domains of this protein. The active site of CDC34 must be located proximal to the targeted lysines in the tail in order for Ub to be transferred; this may be a stable interaction or may occur transiently because of free movement of the polypeptide chain. Autoubiquitination has been proposed to afford a means of regulating CDC34 activity. The tail of UBC6 functions as a membrane anchor and localizes UBC6 to the endoplasmic reticulum membrane with its catalytic domain facing the cytosol. Compartmentalization of UBC6 may determine what proteins are targeted by this E2. Finally, UBC1 has been found to catalyze alternative chain linkages based on the presence or absence of its tail (Hodgins *et al.*, 1996), and this shows that the tail can also participate in the creation of Ub-Ub linkages. These examples demonstrate the functional diversity of the E2 tail domains.

Unlike the tail domains, the catalytic domains, present in all E2s, contain regions of highly conserved residues presumably responsible for either E2 folding or for functions common to all E2s. In contrast nonconserved regions in the catalytic domains may be involved in functions distinct to a particular E2, such as recognition of target proteins or binding of transacting factors responsible for substrate specificity or regulation. Evidence exists to support the notion that catalytic domains contain determinants required for specific E2 functions. Null mutants of class I E2s (those that consist of only the catalytic domain) have distinct phenotypes; therefore these E2s must possess specific determinants that allow them to carry out their unique cellular functions. Evidence also comes from catalytic domain swapping experiments. Fusion of the catalytic domain of RAD6 to the CDC34 tail results in a hybrid protein that is able to complement the loss of cell cycle function of a *cdc34* disruption mutant (Silver *et al.*, 1992). Similar derivatives involving the UBC4 and UBC1 catalytic domains do not have CDC34 activity. RAD6, therefore, contains functional determinants within its catalytic domain in common with CDC34 that are not present in UBC4 or UBC1. Thus E2 catalytic domains possess specific functional information.

Alignment of the protein sequences of the thirteen known yeast E2s reveals four places where insertions of variable lengths exist in some E2s (Cook *et al.*, 1997). These hypervariable regions may contribute to the specificity of an E2. Evidence to support this conclusion comes from reports that a twelve amino acid insert in one E2, CDC34, is necessary for its cell cycle activity (Liu *et al.*, 1995; Pitluk *et al.*, 1995). Removal of the insert does not completely inactivate the protein as the deletion derivative is still able to ubiquitinate itself *in vitro*. This suggests that the insert is involved in CDC34 specificity.

UBC7 contains a similar insert at the same position as CDC34 that, according to the recently solved structure of UBC7, forms a loop beside the active site (Cook *et al.*, 1997). Nevertheless deletion of the insert in CDC34 along with mutation of sequences encoding two other residues, results in a protein that can complement a *cdc34* disruption strain (Liu *et al.*, 1995). Therefore the role of the insert in CDC34 function remains to be clarified.

RAD6 has also been dissected in order to determine the functions of specific regions of its catalytic domain. The N termini of all RAD6 homologues are highly conserved, but this conservation does not extend to other E2s. Sullivan and Vierstra (1991) found that mutation of residues 6-8 in RAD6 decreased Ub thiolester formation to 4% of the wild-type protein, implying that these residues somehow participate in thiolester formation. Watkins and colleagues (1993) created a RAD6 derivative missing the first nine residues (rad6$\Delta_{1\text{-}9}$). This derivative is competent in induced mutagenesis, but shows a reduction in its DNA repair ability and is defective in sporulation and degradation of N end rule pathway substrates. The inability of rad6$\Delta_{1\text{-}9}$ to degrade N end rule substrates could be explained by the fact that it can no longer interact with UBR1, the N end rule specific E3. In addition scanning mutagenesis has been done in which clusters of charged residues in RAD6 were mutated to alanine residues (McDonough *et al.*, 1995). Charged residues are most likely found on the surface of a protein, and their mutation may perturb protein-protein interactions rather than altering protein folding. Once again residues 6-8 were found to be important for RAD6 activity. In addition, changes near the active site (residues 71,75, 86, and 90) created derivatives that were devoid of most RAD6 *in vivo* activities. These derivatives were, however, still able to conjugate Ub to histones indicating that the residues were likely involved in specific functions and not thiolester formation. In contrast to the functions described above, the RAD6 N terminus is not required for its interaction with RAD18, a DNA binding protein involved in DNA repair (Bailly *et al.*, 1994). Because of their differential binding to an N terminally deleted rad6 derivative, UBR1 and RAD18 may form separate complexes with RAD6; the RAD6-UBR1 complex would participate in the

N end rule pathway and RAD6-RAD18 in DNA repair. As RAD6 possesses additional functions that appear not to require UBR1 or RAD18, further dissection of the RAD6 core domain will likely reveal regions involved in novel transacting factor interactions and hopefully lead to the isolation of these factors.

In conclusion, knowing the regions of an E2 that define different E2 functions will aid in the identification of proteins that interact with E2s. Characterization of these binding proteins will begin to resolve how E2s perform their various functions.

The objective of my research was to study the structural basis of E2 function. First I examined the structural requirements of UBC4 ubiquitination *in vivo* and the formation of a UBC4 homocomplex *in vitro*. Next I dissected UBC4 and RAD6 to identify regions important for the specific functions of these E2s. Finally, I attempted to reconstitute in yeast the degradation of a specific target of UBC4, p53.

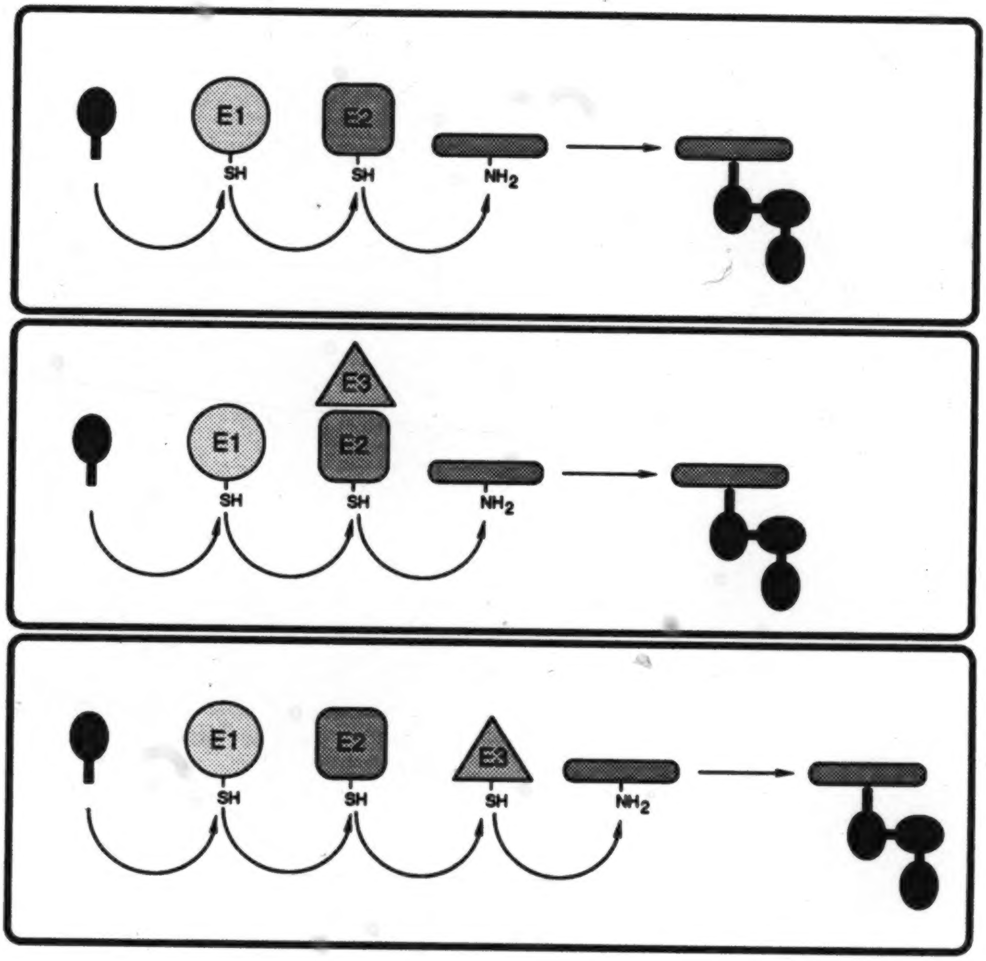

Figure 1: Potential pathways of ubiquitin transfer.

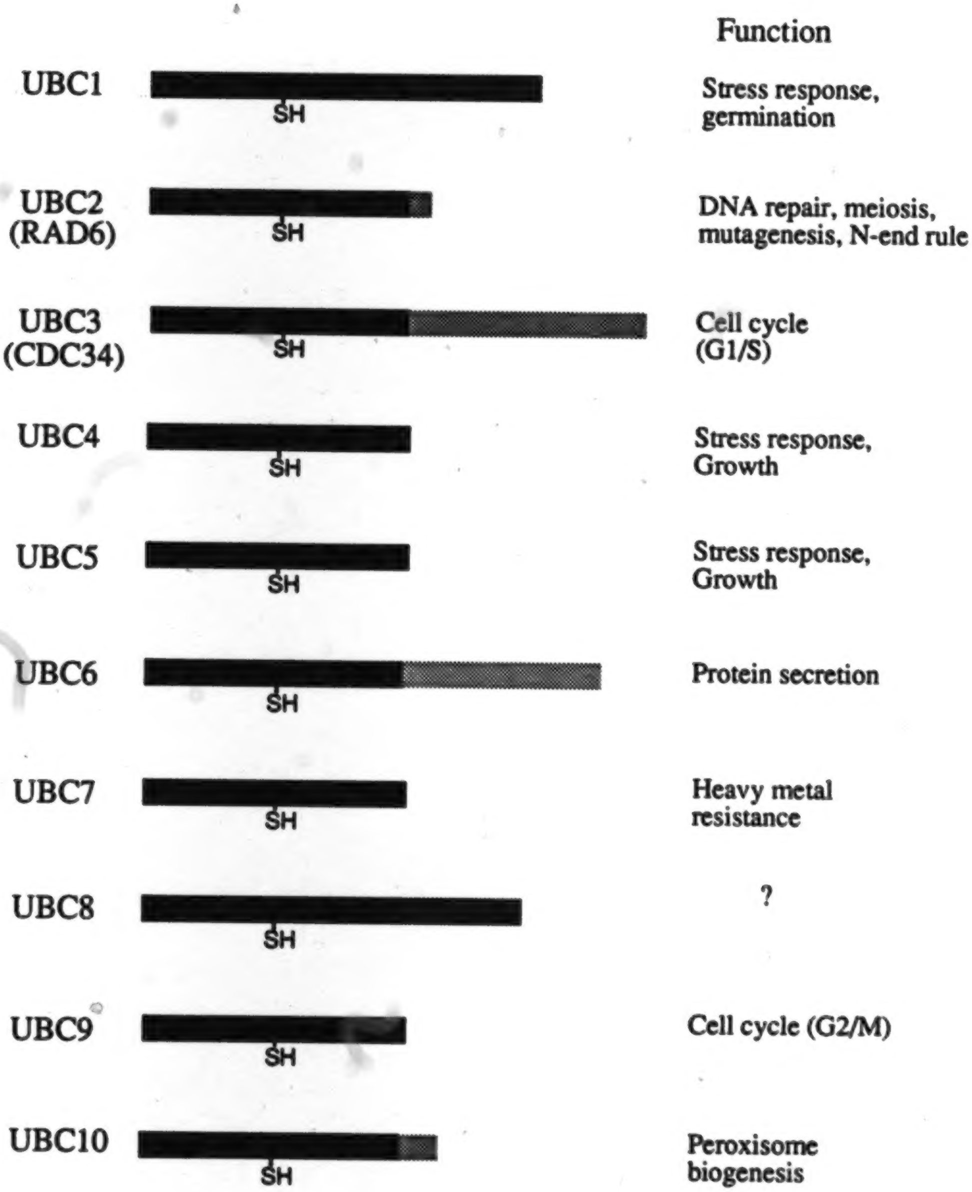

Figure 2: The Yeast Ubiquitin Conjugating Enzymes

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Chapter II

The Yeast UBC4 Ubiquitin Conjugating Enzyme Monoubiquitinates Itself *In Vivo*: Evidence for an E2-E2 Homointeraction[1]

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INTRODUCTION

E2s are a class of eukaryotic proteins that catalyze the transfer of Ub either directly from E1 to the lysines of appropriately targeted proteins, or to E3s which then transfer Ub to proteins. Mutational analyses of E2 genes in yeast have revealed that these enzymes participate in a broad range of cellular processes and therefore play at least an indirect role in target protein recognition. Certain E2s also catalyze the formation of the multi-Ub chain (Chen and Pickart, 1990; Haas *et al.*, 1991; Johnson *et al.*, 1992), a structure whose assembly onto targeted proteins is strongly correlated with their turnover.

Several lines of evidence indicate that E2 molecules interact with one another in homo and hetero complexes and that in certain cases these types of interactions play an important role in E2 function. Three groups have reported for instance that certain E2s can be purified as homodimers and higher order complexes (Pickart and Rose, 1985; Haas and Bright, 1988; Girod and Viestra, 1993). Silver *et al.* (1992) presented genetic evidence suggesting that the cell cycle function of the CDC34 (UBC3) Ub conjugating enzyme was dependent on its interaction with itself or with the DNA repair enzyme RAD6 (UBC2). Ptak *et al.* (1994) demonstrated using cross-linking studies that CDC34 can form multimeric complexes *in vitro* and that this interaction is important *in vivo* for the cell cycle function of CDC34. In addition Chen *et al.* (1993) have found that the turnover of the yeast MATα2 transcriptional regulator is strongly correlated with the interaction of UBC6 and UBC7, and have also presented evidence for the interaction of UBC7 with itself.

The possible functions associated with E2-E2 interactions are not known. Silver *et al.* (1992) have proposed a model based on the interaction of one E2 molecule with another that accounts for the initiating steps in multi-Ub chain synthesis. On the other hand, Chen *et al.* (1993) have suggested that the ability of E2s to interact amongst themselves in different combinations may serve to increase the repertoire of substrate specificities found within the Ub system.

In this chapter further evidence is presented for the interaction of E2 monomers *in vivo*. It has been found that one of the stress related Ub conjugating enzymes, UBC4, monoubiquitinates itself at a single lysine residue in an intermolecular reaction. This observation suggests that UBC4 interacts with itself in a specific manner and provides an important structural landmark for defining the nature of this interaction. Further evidence for UBC4 homointeractions comes from *in vitro* cross-linking studies in which UBC4 monomers can be specifically cross-linked to one another.

MATERIALS AND METHODS

Plasmids and Yeast Strains

High copy *TRP1* yeast expression vectors carrying the *UBC4* gene derivatives shown in Figure 1 are identical to YEp96 (Ellison and Hochstrasser, 1991) except that the *UBC4* coding sequence replaces the *Ub* coding sequence. In all *UBC4* plasmids, the serine-2 codon AGC has replaced the serine-2 codon TCT found in the wild-type sequence (Seufert and Jentsch, 1990) in order to create a *Sac*I site. Lysine to arginine mutations were made by changing the appropriate lysine codon to the arginine codon, AGA. In *ubc4A86*, the active site cysteine-86 codon was replaced with the alanine codon, GCT. The influenza virus hemagglutinin peptide epitope sequence (Wilson *et al.*, 1984), which will be referred to here as the HA tag, was fused to both the amino and carboxy termini of UBC4. N-terminally HA tagged UBC4 (hUBC4) was created by appending a sequence encoding the peptide MYPYDVPDYASLG to the methionine-1 codon of *UBC4* (peptide epitope underlined). C-terminally HA tagged UBC4 (UBC4h) was created by appending a sequence encoding the peptide GYPYDVPDYASLG to the last codon of *UBC4* . The coding sequence of each *UBC4* derivative was verified by DNA sequencing using an Applied Biosystems 373A DNA sequencer operated by the University of Alberta, Department of Biochemistry, DNA Synthesis and Sequencing Laboratory.

Low copy *TRP1* plasmids carrying *UBC4* and its arginine-64 (*ubc4R64*) and arginine-144 (*ubc4R144*) derivatives under the control of the native *UBC4* promoter, were constructed by positioning an expression cassette between the *Cla*I and *EcoR*I sites of pRS314 (Sikorski and Hieter, 1989). Sequences of the cassette which define the *UBC4* coding sequence and downstream *CYC1* terminator sequence (including the flanking *Cla*I site) are identical to the high copy vectors described above. The promoter sequence of *UBC4* extends from -1 to -524 (with a G to T change at -521 to create an *EcoR*I site) and is contiguous to the *EcoR*I site of the vector.

The yeast high copy *URA3* plasmids expressing either Ub or N-terminally myc-tagged Ub (mUb) were created by inserting the *BamHI/Cla*I fragment (containing the *CUP1* promoter, *Ub* or *mUb* coding sequence, and *CYC1* terminator) from either YEp96 or YEp105 (Ellison and Hochstrasser, 1991) into YEp352 (Hill *et al.*, 1986) between the *BamH*I and *Nar*I sites. The high copy *TRP1* negative control plasmid pES12 (Hodgins *et al.*, 1992) is identical to YEp96 except for deletion of the *Ub* coding sequence. The high copy *URA3*-pUB23P plasmid (expressing Ub-pro-β-galactosidase (Ub-pro-β-gal)) and the *URA3*-pUB23M plasmid (expressing Ub-met-β-galactosidase (Ub-met-β-gal)) have been previously described (Bachmair *et al.*, 1986).

The pET-3a-UBC4 plasmid used for the expression and purification of recombinant UBC4 from *Escherichia coli* is identical in sequence to the pET3a-CDC34 plasmids described elsewhere (Ptak *et al.*, 1994) except that the coding sequence for UBC4 (as described above but minus its intron) replaces the coding sequence of CDC34. Details on the construction of all plasmids described here for the first time are available on request.

For Western analysis, selected high copy *TRP1-UBC4* plasmids and the control plasmid, pES12 were introduced into either of the yeast strains SUB60 (Finley *et al.*, 1987) or MHY498 (Chen *et al.*, 1993) in combination with either the *Ub-URA3* or *mUb-URA3* plasmids. SUB60 carries a deletion for the polyUb gene, *UBI4* and has the genotype: *MAT* a, *lys2-801*, *leu2-3*, *leu2-112*, *ura3-52*, *his3-Δ200*, *trp1-1*(am), *ubi4Δ:LEU2*. MHY498 (obtained from M. Hochstrasser) carries a deletion for the *UBC4* gene and has the genotype: *MAT α, his3-Δ200, leu2-3, 112, ura3-52, lys2-801, trp1*-1, *ubc4-Δ1::HIS3*.

For phenotype analysis, low copy plasmids expressing either wild-type UBC4, ubc4R64 or ubc4R144 were introduced into MHY508 (obtained from M. Hochstrasser). MHY508 (Chen *et al.*, 1993) carries a deletion for *UBC4* and *UBC5* and has the genotype: *MAT α, his3-Δ200, leu2-3, 112, ura3-52, lys2-801, trp1-1, ubc4-Δ1::HIS3, ubc5-Δ1::LEU2*).

Steady state levels of Ub-pro-β-gal and Ub-met-β-gal were determined using the above three MHY508 strains cotransformed with either of the pUB23 plasmids or YEp352.

Protein Expression and Western Analysis

Cells were grown to early exponential phase at 30°C in synthetic defined (SD) media (Sherman *et al.*, 1986) supplemented with selected amino acids critical for growth (for SUB60: histidine 10 mg/L, and lysine 40 mg/L; for MHY498: lysine 40 mg/L, and leucine 60 mg/L). Cultures were then diluted to $3x10^6$ cells/ml in the presence of $CuSO_4$ (100 μM) to induce gene expression and were allowed to grow for two additional generations prior to harvesting. Cells were then washed in cold 10 mM $CdCl_2$, 2 mM EDTA (to inhibit proteolysis and isopeptidase activity) followed by resuspension in electrophoresis load mix (12.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% sodium dodecyl sulfate (SDS), 0.001% bromophenol blue, 200 mM dithiothreitol (DTT)). Samples were then boiled (10 minutes) and centrifuged to remove cellular debris. Sample supernatants were electrophoresed on an SDS polyacrylamide gel (18% acrylamide, 0.09% bisacrylamide). For Figure 2, Western analysis was performed as described in Hodgins *et al.* (1992). All other Western analyses were performed as above except that following anti-myc antibody treatment, filters were washed in 1x Tris buffered saline + Tween (TBS+Tween, 10 mM Tris-HCl, pH 7.5, 0.9% NaCl, 0.05% Tween-20) 3 times followed by incubation in 50 ml of TBS+tween containing 17 µl of Goat anti-Mouse IgG Horseradish peroxidase conjugate (BioRad) for 45 minutes at 4°C. Filters were then washed as above. Protein bands bearing the myc epitope were visualized using Enhanced Chemiluminesence (ECL) (Amersham).

Chemical Cleavage Analysis

SUB60 cells coexpressing mUb and either UBC4 or C-terminally HA tagged UBC4 (UBC4h) from high copy vectors were grown and processed in the same way as described for Western analysis. Protein samples from approximately 1x10^8 cells were electrophoresed on an 18% polyacrylamide SDS gel. Following electrophoresis, mUb-UBC4 and mUb-UBC4h conjugates were excised from the gel based on their known migration relative to pre-stained molecular weight markers. Protein was electroeluted from the acrylamide slices using the procedure of Hunkapiller *et al.* (1983). Formic acid was added to the protein solution to 70% and the reaction was incubated at 37°C for 24 hours. BSA was then added as a carrier and the reaction was extracted using the methanol chloroform procedure of Wessel and Flügge (1984). The resulting protein precipitate was boiled in electrophoresis load mix and Western analysis was performed as outlined above.

UBC4 Expression and Purification

The pET-3a-UBC4 expression plasmid was co-transformed into the *E. coli* strain BL21 in combination with the thermally inducible T7 polymerase plasmid, pGP1-2 (Tabor and Richardson, 1985). Cells were grown in LB liquid media (containing 50 µg/ml ampicillin and 40 µg/ml kanamycin) to an absorbance of 0.4 at 590 nm. Cells were washed two times in cold M9 media and then grown for 2 hours at 30°C in 25 ml of M9 media supplemented with 1 mM $MgS0_4$, 0.1 mM $CaCl_2$, 12 mM glucose, 18 µg/ml thiamine, 50 µg/ml ampicillin, 40 µg/ml kanamycin and all amino acids (40 µg/ml) except for cysteine and methionine. Cultures were transferred to 42°C for 40 minutes followed by the addition of rifampicin (200 µg/ml final). Cells were incubated for 30 minutes at 42°C followed by a shift to 30°C for 20 minutes. *Trans*-[^{35}S]-methionine (ICN) was then added (25 µCi/ml) followed by incubation for 40 minutes at 30°C. Cells were harvested by centrifugation, resuspended in 250 µl of 25% sucrose, 50 mM Tris-Cl (pH 8.0) and lysed with lysozyme as previously described (Gonda *et al.*, 1989). Labeled UBC4 was purified using an FPLC system (Pharmacia Biotech Inc.). Clarified supernatants in 20 mM Hepes, pH 6.9, 1 mM DTT were passed over a Mono S HR 5/5 ion exchange column (Pharmacia) equilibrated with the same buffer. The flow through was collected and then passed over a MonoQ HR 5/10 ion exchange column (Pharmacia), equilibrated with 50 mM Tris HCl, pH 7.5, 1 mM DTT and eluted with a NaCl gradient from 0 mM to 1 M. Under these conditions, UBC4 eluted in essentially pure form as a major protein peak at 60 mM NaCl.

Protein Cross-linking

Prior to cross-linking, UBC4 was dialyzed into cross-linking buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM DTT). Aliquots (40 µl) were preincubated on ice for 5 minutes, followed by the addition of 0.1 volume of the cross-linker BS^3 [bis(sulfosuccinimidyl)suberate (Pierce)] in cross-linking buffer. Samples were incubated an additional 30 minutes on ice. BS^3 was added to a final concentration of 0, 0.4, or 10 mM to reactions that contained ^{35}S-labeled UBC4 (6.6 µM, 1.6x10^4 cpm/µM) in the presence (66 µM) or absence of Bovine Serum Albumin (BSA). Cross-linked species were detected by SDS-polyacrylamide gel electrophoresis (16%) followed by autoradiography.

Phenotype Analysis

For stress sensitivity experiments MHY508 strains were grown in SD media supplemented with uracil (40 mg/L) and lysine (40 mg/L) as described above. Appropriate dilutions of early exponential cultures were then spread onto supplemented SD plates with or without canavanine (1.5 µg/ml) and grown at 30°C. Cultures were also streaked onto supplemented SD plates and grown at 38°C. For β-galactosidase experiments, cells were grown to a final density of 5x10^7 cells/ml in liquid media containing 0.7% Difco yeast nitrogen base, without amino acids, 2% galactose, 2% glycerol, 2% ethanol, plus lysine (40 mg/ml). β-galactosidase activity was measured as previously described (Reynolds and Lundblad, 1989).

RESULTS

UBC4 is Monoubiquitinated *In Vivo*

In previous work it has been shown that the expression of mUb in yeast could be a useful tool for detecting Ub-protein conjugates formed *in vivo* (Ellison and Hochstrasser, 1991). Typically, these tagged conjugates were detected by SDS-polyacrylamide gel electrophoresis (PAGE) of total yeast protein followed by Western analysis using an anti-myc epitope antibody. Interestingly, when this analysis was applied to cells overexpressing the stress Ub conjugating enzyme UBC4 from a high copy plasmid, a prominent band of approximately 25 kDa was detected that rivaled the levels of free mUb (Figure 2). The absence of this band in cells expressing UBC4 in combination with unmodified Ub establishes that mUb is present in this species and that the band is not another protein that cross reacts with the anti-myc antibody. In addition the absence of the band in cells expressing only mUb (not UBC4) demonstrates that this species is formed in a UBC4 dependent manner. Based on the above observations and the molecular weight of mUb (approximately 10 kDa) it can be concluded that the 25 kDa species is a mUb protein conjugate in which mUb is coupled in a UBC4 dependent manner to another protein, of approximately 15 kDa in molecular weight.

It was possible that the 15 kDa component of the conjugate was an abundant substrate for UBC4 dependent ubiquitination, and that the steady state levels of the conjugate increased dramatically when UBC4 was overexpressed. Another possibility, however, was that the conjugate was composed of mUb and UBC4 itself, an idea that is consistent with both its abundance and molecular weight. This idea was tested by altering the molecular weight of UBC4 (approximately 1.4 kDa) by appending the HA peptide tag to its amino terminus. If the mUb conjugate contains UBC4 then expression of tagged UBC4 should shift the electrophoretic position of the mUb conjugate upward by a corresponding amount. If however UBC4 is not the target of ubiquitination, then the position of the mUb conjugate will remain unaltered. As indicated in Figure 2, the decreased mobility of the mUb conjugate from cells expressing tagged UBC4 relative to cells expressing untagged UBC4 demonstrates that the major conjugate formed under these conditions consists of a single molecule of mUb coupled to UBC4.

It was possible that the mUb-UBC4 conjugate was actually a thiol ester intermediate formed by the transfer of mUb by the Ub activating enzyme (E1) to cysteine-86 of UBC4. This possibility seemed unlikely in view of the acknowledged instability of Ub-E2 thiol esters and in light of the fact that the protein samples were boiled in 200mM DTT prior to electrophoresis. However in order to definitively rule out this possibility, a UBC4 derivative that was defective in thiol ester formation was constructed in which cysteine-86 was replaced with alanine. The observation that this derivative was still targeted for ubiquitination (Figure 2), indicated that the mUb-UBC4 conjugate was not a thiol ester intermediate and that mUb was being conjugated to some other site, most probably a lysine. Significantly this result also demonstrated that the ubiquitination of UBC4 does not occur by an intramolecular reaction mechanism in which mUb is passed from the active site of UBC4 to a site on the same molecule. Instead, the ubiquitination of UBC4 must be catalyzed by the transfer of mUb to UBC4 by another E2 molecule.

Ubiquitination of UBC4 Occurs at a Single Lysine

To determine which lysine in UBC4 is targeted for ubiquitination, a series of *UBC4* gene derivatives were created that carried lysine to arginine codon substitutions at different positions within the *UBC4* coding sequence. If a single lysine within UBC4 served as the site of ubiquitination, then mutation of this residue would result in the complete disappearance of the UBC4 conjugate. When cells expressing the various UBC4 mutants were subjected to the same analysis as described for Figure 2, it was found that lysine to arginine substitutions at either position 64 or 144 result in the disappearance of the UBC4 conjugate while lysine to arginine substitutions at other positions had no effect (Figure 3). If both positions, 64 and 144, served as targets for ubiquitination then substitution at one position would still leave the other available for mUb attachment and would result in only a partial reduction in UBC4 conjugate levels. The complete disappearance of the UBC4 conjugate in either case however, argues that one lysine is targeted for ubiquitination while the other lysine fulfills an indirect but essential role in the reaction.

A chemical cleavage strategy was used to discern which of the two lysines, K64 or K144, was ubiquitinated (Figure 4A). UBC4 contains two Asp-Pro peptide bonds that are sensitive to cleavage upon treatment with formic acid. One of these peptide bonds is situated between K64 and K144 at position 118. Cleavage of mUb-UBC4 with formic acid would give rise to three fragments whose molecular weights depend upon whether mUb was positioned at K64 or K144 (Ub does not contain an acid sensitive site). By comparing the electrophoretic cleavage pattern of purified conjugates formed from either UBC4 or C-terminally HA-tagged UBC4 (UBC4h), ubiquitination at K64 or K144 can be assigned unambiguously. The positioning of mUb at K64 for instance would give rise to a fragment of approximately 21 kDa (fragment B + mUb) common to both the UBC4 and tagged UBC4 conjugates. The positioning of mUb at K144 would however give rise to a smaller fragment of approximately 14 kDa for the UBC4 conjugate which would shift to approximately 15 kDa upon addition of the tag (fragment C*+mUb). Furthermore, only those fragments coupled to mUb can be visualized using the anti-myc antibody as a probe. As seen in Figure 4B, the addition of the HA tag to UBC4 results in a corresponding increase in the molecular weight of the smallest fragment coupled to mUb. Based on this result it can be concluded that UBC4 is ubiquitinated at K144.

UBC4 Monoubiquitinates Itself

The observation that the active site mutant of UBC4 (ubc4A86) is ubiquitinated *in vivo* indicated that mUb was being transferred to UBC4 in an intermolecular reaction catalyzed by one of potentially any of the E2s found in yeast including UBC4 and its homologue UBC5. In principle, the involvement of either UBC4 or UBC5 in this reaction was best resolved by examining whether or not ubc4A86 was ubiquitinated in a strain of yeast deleted for both the *UBC4* and *UBC5* genes. In practice, expression of ubc4A86 in the double mutant proved to be toxic (results not shown). In contrast, cells expressing ubc4A86 in a strain deleted only for *UBC4* were viable. The results of Figure 5 illustrate that when UBC4 and ubc4A86 are expressed to similar levels in the *UBC4* deleted strain (right panel), UBC4 is ubiquitinated whereas ubc4A86 is not (left panel). Based on these results, it can be concluded that UBC4 ubiquitinates itself. The strong dependence of this ubiquitination on UBC4 activity, inspite of the presence of the *UBC5* gene, can be explained by the previous observation that under exponential growth conditions, UBC5 expression is low when compared to UBC4 (Seufert and Jentsch, 1990).

Nonubiquitinatable ubc4 Derivatives Have No Obvious Phenotype

Double deletion mutants of *UBC4* and *UBC5* display a variety of phenotypes including slow growth, and extreme sensitivity to chronic heat stress and to growth on the amino acid analogue canavanine (Seufert and Jentsch, 1990). In addition *UBC4* single mutants show reduced turnover rates for the yeast MATα2 repressor (Chen *et al.*, 1993), and for the noncleavable fusion protein Ub-pro-β-gal (Johnson *et al.*, 1992).

If the ubiquitination of UBC4 facilitated any of the processes reflected by the above phenotypes, then the nonubiquitinatable mutants ubc4R64 and ubc4R144 might be expected to display some or all of these defects. This possibility was tested by introducing low copy plasmids for *UBC4*, *ubc4R64* and *ubc4R144* (driven by the native *UBC4* promoter) into the *ubc4ubc5* double mutant and assessing their growth and viability under two forms of environmental stress: canavanine sensitivity and chronic heat stress. It is clear from Table 1 that mutations at positions 64 and 144 have no detrimental effect on the ability of cells to survive exposure to either stress. In addition cells carrying these mutations display characteristically normal growth rates relative to cells carrying wild-type *UBC4* (data not shown).

In another experiment the effect of the R64 and R144 mutations on the turnover of Ub-pro-β-gal was examined by measuring the steady state levels of Ub-pro-β-gal in *ubc4ubc5* mutant cells carrying the plasmids described above. Johnson *et al.* (1992) have previously shown that the metabolic instability of Ub-pro-β-gal is UBC4 dependent and results from the recognition of the Ub portion of the fusion as a degradation signal. While there appears to be slight stabilization of Ub-pro-β-gal in R64 and R144 mutants (Table 2), normal variation in β-galactosidase determinations makes it impossible to attach significance to these differences. Thus it would appear that the ubiquitination of UBC4 *in vivo* is not essential to any of the UBC4 dependent processes examined above. Although there may be other subtle phenotypes associated with UBC4 for which K144 ubiquitination is important, the significance of this result lies in its inference that UBC4 is capable of interacting with itself *in vivo*.

To prove that UBC4 does indeed interact with itself, an *in vitro* cross-linking study was done in which purified radiolabeled UBC4 was incubated in the presence of the amino group specific cross-linker BS^3 (Figure 6). The addition of the cross-linker to UBC4 resulted in the appearance of two UBC4 cross-linked species with apparent molecular weights of 36 kDa and 47 kDa. These values are consistent with the molecular weights predicted for a UBC4 dimer (32 kDa) and a UBC4 trimer (48 kDa) respectively. The reduced intensities of these species at the higher cross-linker concentration probably reflects a reduction in the number of unmodified lysine residues that are available to complete the cross-link. The fact that the addition of a 10-fold molar excess of BSA to the UBC4 cross-linking reaction (as a non specific negative control) neither significantly decreased the yield of these species nor resulted in the appearance of new UBC4-BSA cross-linked species, indicates that the cross-linking of UBC4 to itself reflects a specific interaction between UBC4 monomers *in vitro*. The apparent reduction in the molecular weight of UBC4 trimer in the presence of BSA is an artifact of the high BSA concentration in this region of the gel.

DISCUSSION

The present study demonstrates that the yeast Ub conjugating enzyme UBC4 is monoubiquitinated *in vivo* in a highly specific intermolecular reaction that involves a single target lysine (K144), and that depends upon the presence of active UBC4. The observation that Ub cannot be transferred from the E2 active site to the target lysine (K144) of the same molecule is easily rationalized by the distance that separates these two residues, as indicated on the three dimensional structure of UBC4 (Figure 7) originally determined by Cook *et al.* (1993).

The trivial explanation that a fraction of the UBC4 expressed in yeast is either damaged or abnormal and is therefore targeted by the Ub dependent proteolytic system for degradation, can be ruled out on the basis of three observations. First, the monoubiquitination of UBC4 is clearly atypical of other substrates that are degraded in a Ub dependent manner since it lacks the multi-Ub chain that facilitates proteolysis. Secondly, UBC4 displays no obvious sign of metabolic instability (unpublished observation). Finally, the observation that UBC4 ubiquitination is eliminated by substitution of a single surface lysine (K64) that is distally situated with respect to the target lysine, without apparent loss of UBC4 function, is difficult to explain using conventional notions of protein damage or abnormality.

The simplest, most straightforward explanation for the ubiquitination of UBC4 is that one monomer of UBC4 transfers Ub to the target lysine of another UBC4 monomer via a direct and specific interaction that positions the active site of one monomer close to K144 of the other monomer. This conclusion is based on the observations that the reaction is intermolecular and both requires a UBC4 monomer as an acceptor of Ub and a UBC4 monomer as a donor of Ub. It is also based on the specific interaction of UBC4 monomers that is detected *in vitro* by cross-linking analysis. If the interpretation of UBC4 ubiquitination is correct, then the necessity of positioning the active site of one monomer nearby K144 of the other monomer provides important structural information for determining the geometrical organization of both Ub and UBC4 within the dimeric complex, particularly in view of the known structures for Ub (Vijay-Kumar, *et al.*, 1987), the K48 linked Ub dimer (Cook *et al.*, 1992) and UBC4 (Cook *et al.*, 1993).

The interaction of UBC4 with itself which is inferred from its ubiquitination *in vivo* and which has been demonstrated directly by cross-linking analysis, strengthens the viewpoint that E2-E2 interactions constitute a general phenomenon. Based on the degree of evolutionary conservation exhibited by all E2s identified to date, it is reasonable to expect that these E2-E2 interactions whether they are of the homo variety or the hetero variety, will ultimately conform to a common structural theme.

Several indirect pieces of evidence suggest that the monoubiquitination of UBC4 is facilitated by the interaction of UBC4 with another factor, possibly a ubiquitin protein ligase (E3). First, although the UBC4-UBC4 interaction detected *in vitro* is specific, an inability to detect formation of the homodimer by gel exclusion chromatography (results not shown) indicates that it is also weak. This observation, coupled with the fact that K144 is not targeted for ubiquitination *in vitro* using purified components (results not shown), may indicate that either another protein stabilizes the UBC4-UBC4 interaction sufficiently for the transfer of Ub from one UBC4 monomer to the other to occur *in vivo*, or positions K144 in a conformation that is favorable for ubiquitination. Secondly, the observation that UBC4 does not serve as a substrate for multi-Ub chain assembly is clearly atypical of most if not all *in vivo* substrates of ubiquitination reported thus far. The fact that even free Ub functions as a substrate for the further additon of Ub (Chen and Pickart, 1990) would suggest that UBC4 is part of a complex whose subunit geometry precludes the possibility of multi-Ub chain assembly at K144. Finally the observation that UBC4 ubiquitination is eliminated by mutation of K64, a lysine that is situated on the E2 surface far removed from the ubiquitinated position, suggests that K64 could be involved in stablizing the interaction of UBC4 with a trans acting factor that is necessary for UBC4 ubiquitination (although stabilization of the UBC4-UBC4 interaction by K64 cannot be ruled out). While the identity of this putative factor is not known, the site specific ubiquitination of UBC4 may prove to be an effective biochemical assay for its purification.

An alternative mechanism of UBC4 ubiquitination, that cannot be ruled out, is that the ultimate donor of Ub to UBC4 is an E3 rather than UBC4 itself. Several E3s are able to form a Ub thiolester conjugate (Hochstrasser, 1995; Huibregtse *et al.*, 1995; Rolfe *et al.*, 1995; Scheffner *et al.*, 1995), and for one E3 it has been demonstrated that Ub can be transferred from the E3 directly to a target protein (Rolfe *et al.*, 1995; Scheffner *et al.*, 1995). Thus UBC4 ubiquitination may occur through a thiolester cascade in which Ub is transferred from the UBC4 donor molecule to an E3 and finally to the UBC4 acceptor molecule. In this case the E3 may either bind both UBC4 molecules at once or sequentially. The resolution of the mechanism of UBC4 ubiquitination awaits the isolation of the putative E3.

The function of UBC4 ubiquitination is uncertain. As pointed out above, UBC4 displays no obvious signs of metabolic instability; therefore if UBC4 monoubiquitination fulfills an autoregulatory function it is by a mechanism based on the modification of UBC4 and not its degradation. Furthermore, mutations of UBC4 that fail to become ubiquitinated have no adverse effect on the growth or stress related functions of UBC4 or on the UBC4 dependent turnover of Ub-pro-β-gal. Therefore the biological ramifications of this specific reaction remain to be addressed.

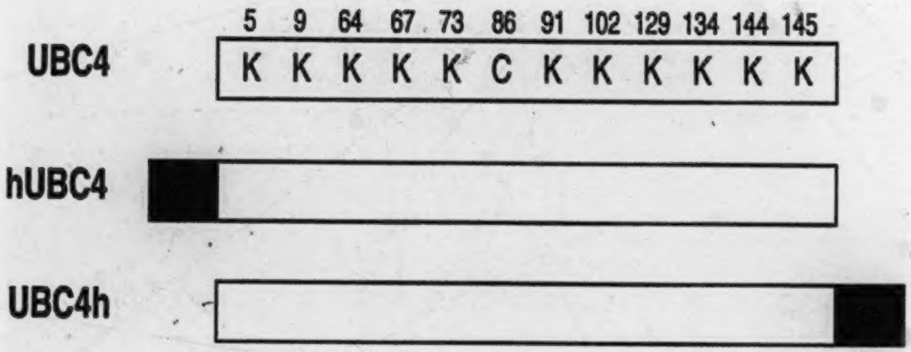

Figure 1: UBC4 derivatives. Open box represents the peptide sequence of UBC4. Black boxes represent the HA epitope. Also shown are the amino acid positions that were changed (K to R, C to A) to produce the mutated UBC4 derivatives.

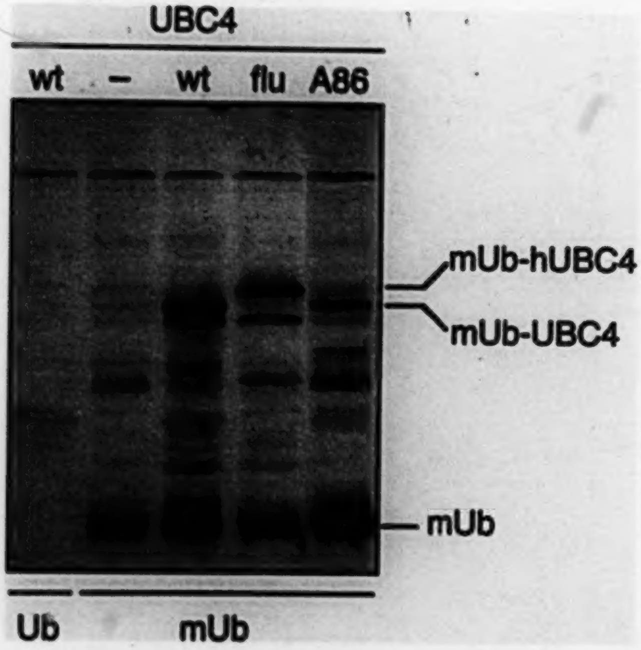

Figure 2: Monoubiquitination of UBC4 *in vivo*. Immunoblot of an SDS gel (probed with the anti-myc antibody) of total protein from yeast expressing either wild-type UBC4 (wt), a negative control plasmid (-), N-terminally HA-tagged UBC4 (flu or hUBC4), or ubc4A86 (A86) in combination with either ubiquitin (Ub) or N-terminally myc-tagged ubiquitin (mUb). The positions of mUb and the mUb-UBC4 and mUb-hUBC4 conjugates are shown. The assignment of molecular weights was made on the basis of migration with respect to known molecular weight markers.

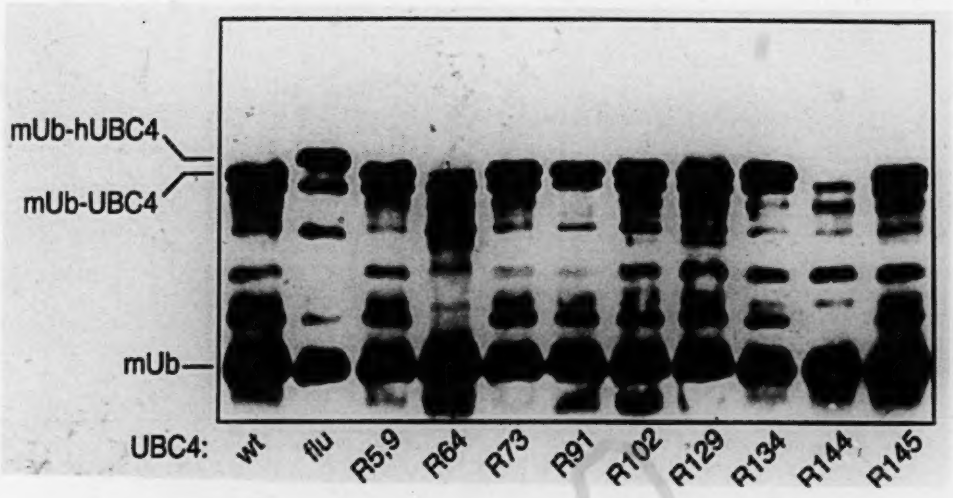

Figure 3: Mapping the site of UBC4 ubiquitination by mutation. Immunoblot of an SDS gel (probed with the anti-myc antibody) of total protein from yeast expressing wild-type UBC4 (wt), N-terminally HA-tagged UBC4 (hUBC4) or one of the UBC4 arginine mutants (R5-R145) in combination with N-terminally myc-tagged ubiquitin (mUb). The positions of mUb and the mUb-UBC4 and mUb-hUBC4 conjugates are shown.

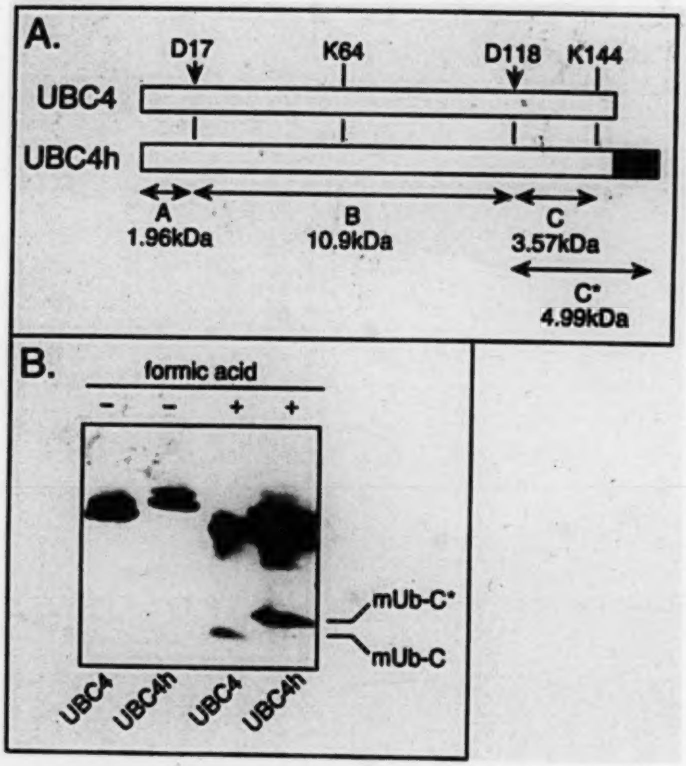

Figure 4: Chemical mapping of the UBC4 ubiquitination site to K144. A) Schematic of mapping strategy showing the two formic acid cleavage sites (D17 and D118) in UBC4 and UBC4h and the resulting fragments. B) Immunoblot of an SDS gel (probed with the anti-myc antibody) showing the formic acid cleavage pattern of the mUb-UBC4 conjugate (UBC4) or the mUb-UBC4h conjugate (UBC4h). mUb-C and mUb-C* mark the positions of the myc-ubiquitinated forms of the C fragments illustrated in A.

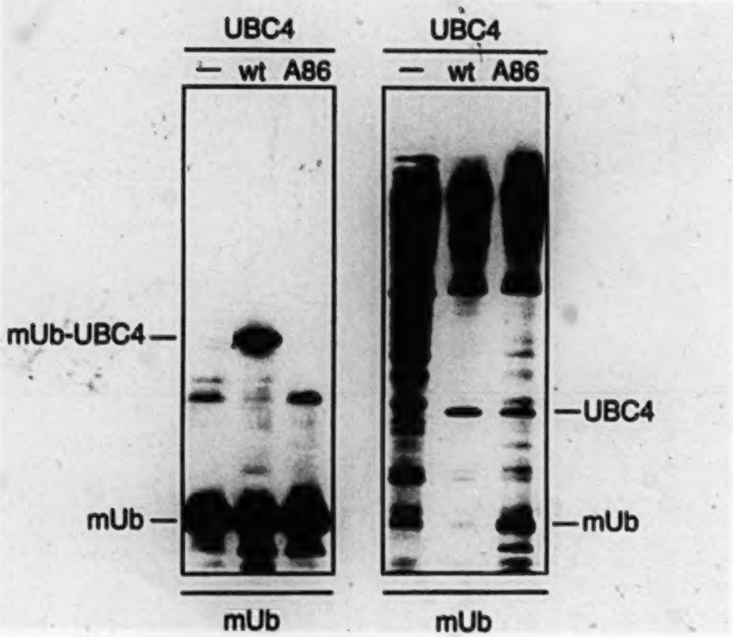

Figure 5: UBC4 monoubiquitinates itself in an intermolecular reaction. Total yeast protein from *ubc4* null cells labeled with [^{35}S] methionine and expressing mUb alone (-) or in combination with UBC4 (wt) or ubc4A86 (A86). Left panel-An immunoblot of an SDS gel probed with the anti-myc antibody. Right panel-An autoradiograph of an SDS gel.

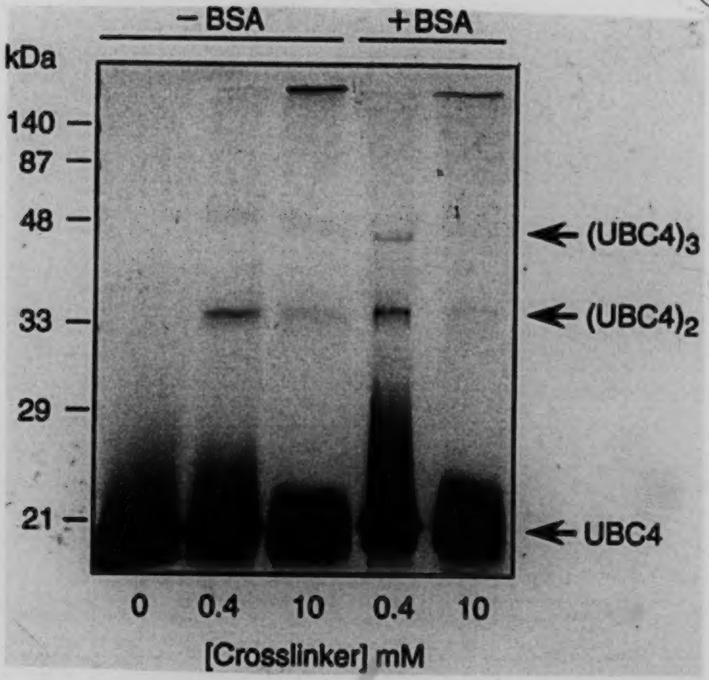

Figure 6: UBC4 can be cross-linked to itself. An autoradiograph of purified radiolabeled UBC4 incubated in the presence or absence of cross-linker and in the presence or absence of a 10-fold molar excess of BSA. The migrations of monomeric (UBC4), dimeric $(UBC4)_2$, and trimeric $(UBC4)_3$ species are indicated relative to molecular weight standards.

Figure 7

Three dimensional space-filling image of UBC4 showing the positions of the active site cysteine (C86), the ubiquitinated lysine (K144), and the lysine affecting ubiquitination (K64). The image was reconstructed from the crystallographic coordinates of UBC4 (Cook *et al.*, 1993) using the molecular modeling package Insight II (Biosym/MSI, San Diego).

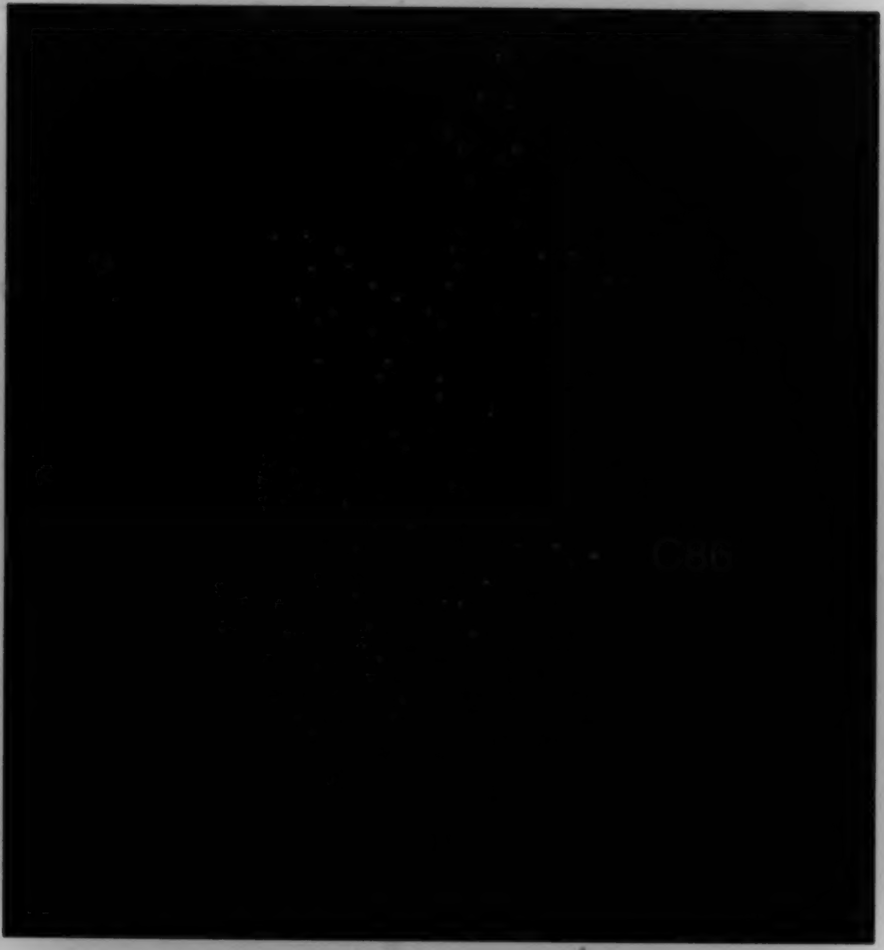
C86

Table 1

UBC4	Resistance to canavanine (%)	Growth at 38°C
wt	69	+
R64	69	+
R144	62	+

Stress phenotypes of non-ubiquitinated ubc4 mutants. The *ubc4ubc5* double mutant, MHY508, expressing wild-type UBC4 (wt), or one of the non-ubiquitinated ubc4 mutants (R64 and R144), was examined for canavanine and heat sensitivity as described in the Experimental Procedures. Resistance to canavanine is calculated as the number of colonies present on plates containing canavanine relative to the number of colonies on plates lacking canavanine. + and - indicate growth or no growth, respectively, at 38°C.

Table 2

UBC4 plasmid	Units of β-galactosidase activity control	Ub-pro-β-gal	Ub-met-β-gal
wt	7	300	1600
R64	9	410	1300
R144	8	520	1300

Steady state levels of Ub-X-β-galactosidase in non-ubiquitinated ubc4 mutants. β-galactosidase activity was measured for the *ubc4ubc5* double mutant, MHY508, expressing wild-type UBC4 (wt) or one of the non-ubiquitinated ubc4 mutants (R64, R144), in combination with either a control plasmid (pES12), Ub-pro-β-galactosidase (Ub-pro-β-gal) or Ub-met-β-galactosidase (Ub-met-β-gal).

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Chapter III

Identification of Functional Determinants in the Ubiquitin Conjugating Enzymes UBC4 and RAD6

INTRODUCTION

Thirteen E2s have been identified in the yeast *S. cerevisiae*, and these E2s are involved in a wide variety of cellular functions. From *in vitro* work it was originally thought that E2s donate Ub directly to target substrates with additional proteins, referred to as E3s, being required to facilitate target recognition. The view that E2s are the ultimate donors of Ub to substrate proteins has been challenged recently by the discovery that an E3, E6AP, can form a thiolester with Ub and can transfer Ub directly to substrate proteins (Rolfe *et al.*, 1995 and Scheffner *et al.*, 1995). So what is the relative involvement of E2s in substrate selection? E2s may act indirectly by binding specific E3s and charging them with Ub. In this scenario E3s ultimately select the target proteins; this would require the presence of a great number of E3s. However it is possible that E2s still contribute directly to substrate selection, and the combinatorial interactions of E2s and E3s are responsible for target diversity. One further level of target diversity may also be obtained by the ability of E2s to form homo and heterodimers with each other (Pickart and Rose, 1985; Haas and Bright, 1988; Silver *et al.*, 1992; Chen *et al.*, 1993; Girod and Vierstra, 1993; Ptak *et al.*, 1994). Thus functional determinants of target selection may include E2-target interactions, E2-E3 interactions, or E2-E2 interactions.

All E2s contain a conserved 150 amino acid catalytic domain and some E2s have additional tails domains that in certain cases are involved in substrate selectivity. Not all E2s have tails; therefore functional discrimination must also reside within the catalytic domain. RAD6 provides an interesting example of how functional determinants can be distributed in an E2. RAD6 possesses a short acidic tail that is required for only some of its activities including sporulation (Morrison *et al.*, 1988) and amino end rule pathway function (Madura *et al.*, 1993). Derivatives lacking the tail domain are still competent for other RAD6 activities: specifically DNA repair and ultraviolet (UV) induced mutagenesis. Thus functional information can be found throughout an E2.

Exactly what structural determinants of the E2 catalytic domain are required for substrate or trans-acting factor interactions is largely unknown. It was therefore of interest to identify these residues and use this information to map regions on the E2 catalytic domain structure involved in specificity.

MATERIALS AND METHODS

Plasmids and Yeast Strains

The yeast high copy *TRP1* E2 expression vectors used in the growth comparison and UV sensitivity studies are identical to YEp96 (Ellison and Hochstrasser, 1991) except that the $rad6\Delta_{459}$, $rad6\Delta_{450}$ and *UBC4* coding sequences replace the *Ub* coding sequence. A *Sac* I site was created at the 5' end of the coding sequences of $rad6\Delta_{459}$ and $rad6\Delta_{450}$ by replacing the serine-2 codon TCC with the serine codon AGC and the threonine-3 codon ACA with the serine codon TCA. An *Eco*R V site was created at the 3' end of $rad6\Delta_{459}$ by changing the methionine-153 codon ATG to the isoleucine codon ATC. A *Sac* I site was also created at the 5' end of the coding sequence of UBC4 by replacing the serine-2 codon TCT with the serine codon AGC. Additional changes made to the $rad6\Delta_{459}$ and $rad6\Delta_{450}$ sequences, that aided in the construction of other derivatives, included: the substitution of the glycine-51 codon GGA with the glycine codon GGC and the threonine-52 codon ACT wtih the alanine codon GCC to create a *Kas* I site. The proline-98 codon CCA was changed to the proline codon CCC and the threonine-99 codon ACA was changed to the glycine codon GGG to create a *Sma* I site. These changes did not affect the DNA repair activity of $rad6\Delta_{459}$.

The following codons were changed to create the various *rad6Δ* derivatives: arginine codon 8 AGG to the glutamine codon CAG, phenylalanine codon 13 TTT to the leucine codon TTG, arginine codon 15 CGT to the aspartate codon GAT, valine codon 24 GTA to the cysteine codon TGT, asparagine codon 32 AAC to the aspartate codon GAC, asparagine codon 37 AAC to the glutamine codon CAG, arginine codon 54 AGG to the phenylalanine codon TTC, glutamate codon 61 GAA to the threonine codon ACA, asparagine codon 65 AAT to the phenylalanine codon TTT, leucine codon 73 TTG to the threonine codon ACT, glutamate codon 75 GAA to the lysine codon AAA, tyrosine codon 82 TAT to the asparagine codon AAC, glutamine codon 93 CAG to the lysine codon AAG, tyrosine codon 100 TAT to the leucine codon TTG, aspartate codon 101 GAT to the threonine codon ACT, threonine codon 107 ACA to the leucine codon TTA, glutamine codon 110 CAA to the cysteine codon TGT, serine codon 120 TCG to the aspartate codon GAT, asparagine codon 123 AAC to the valine codon GTC, aspartate codon 132 GAT to the threonine codon ACT, threonine codon 144 ACG to the tryptophan codon TGG, valine codon 145 GTA to the threonine codon ACA, serine codon 148 TCT to the tyrosine codon TAT, and tryptophan codon 149 TGG to the alanine codon GCA. In addition, for rad6Δ_{153}+UBC4 N terminus the first ten codons of *rad6*Δ_{459} were replaced with the following sequence encoding the first eight residues of UBC4: ATG AGC TCA TCA AAA AGG ATT GCA. Arginine codon 6 AGG was also mutated in this sequence to the glutamine codon CAG. All the constructs were rad6Δ_{153} derivatives except rad6Δ_{150} F54 T73 K75 W144 T145 Y148 A149, rad6Δ_{150} D120 and rad6Δ_{150} V123.

The following changes were made in the UBC4 coding sequence: phenylalanine codon 63 TTC was changed to the asparagine codon AAC, asparagine codon 80 AAT was changed to the tyrosine codon TAT, aspartate codon 118 GAT was changed to the serine codon TCT, and valine codon 121 GTA was changed to the asparagine codon AAT. In addition the first eight codons of *UBC4* were replaced with the following sequence encoding the first ten residues of RAD6: ATG AGC TCT CCA GCT CGT AGA CGT CTT ATG.

All the changes were created using PCR (polymerase chain reaction) directed mutagenesis, with the sequences verified by DNA sequencing using an Applied Biosystems 373A DNA sequencer operated by the University of Alberta, Department of Biochemistry, DNA Synthesis and Sequencing Laboratory.

Low-copy *TRP1* yeast expression vectors used in the determination of cell doubling times, carried *UBC4*, *rad6*Δ_{459} or a derivative, and were constructed by positioning the *Cla* I/*Bam*H I fragment of the plasmids described above between the *Cla* I and *Bam*H I sites of the CEN/ARS plasmid pRS314 (Sikorski and Hieter, 1989). The fragment carries the CUP1 promoter, E2 coding sequence and downstream CYC1 terminator sequence.

The pET3a-rad6Δ_{153} plasmid used for the expression and purification of recombinant rad6Δ_{153} from *Escherichia coli* (*E. coli*) is identical in sequence to the pET3a-CDC34 plasmids described elsewhere (Ptak *et al.*, 1994) except that the coding sequence for rad6Δ_{153} (as described above but with Arg codons 6, 7 and 11 changed from AGA to CGA and Arg codon 8 changed from AGG to CGG) replaces the coding sequence of CDC34. Details on the construction of all plasmids described here for the first time are available on request.

For growth and canavanine sensitivity studies, plasmids were introduced into the *ubc4ubc5* deleted strain MHY508 (obtained from M. Hochstrasser). MHY508 (Chen *et al.*, 1993) has the genotype MATα, *his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, ubc4-Δ1::HIS3, ubc5-Δ1::LEU2*. For the DNA repair studies, plasmids were introduced into the rad6 deleted strain KMY20 (obtained from K. Madura). KMY20 has the genotype *MATa ade2-1, his3-832, trp1-289, ura3-52, rad6Δ::URA3.*

Phenotype Analysis

For the growth studies MHY508 strains containing the high copy *TRP1* vectors were grown in synthetic defined (SD) media (Sherman *et al.*, 1986) supplemented with uracil (40 mg/L) and lysine (40 mg/L). Cultures in exponential phase were streaked onto supplemented SD plates and grown at 30°C. Growth was scored based on colony size. Colonies were given a designation from + to ++++++ based on their relative sizes compared with colonies expressing rad6Δ_{153} or UBC4 respectively.

For determination of cell doubling times MHY508 strains containing the low copy *TRP1* vectors were grown at 30°C in supplemented SD media. Aliquots were taken regularly throughout exponential phase and cell numbers were determined directly using a Coulter Counter (Model ZF).

For stress sensitivity experiments MHY508 strains were grown in supplemented SD media. Appropriate dilutions of early exponential phase cultures were then spread onto supplemented SD plates with or without canavanine (1.5 mg/ml) and grown at 30°C. Colonies were counted after seven days.

For UV resistance experiments KMY20 strains were grown in SD media containing adenine (20 mg/L) and histidine (10 mg/L). The cultures were appropriately diluted into YPD (1% yeast extract, 2% bactopeptone and 2% glucose) and then spread onto YPD plates. The plates were then irradiated with 72 J/m2 short wave UV light (254 nm).

Protein Expression and Purification

Expression vectors containing the rad6Δ_{153}, rad6Δ_{153} D120 V123, Ub (Hodgins *et al.*, 1996) or wheat E1 (Hatfield *et al.*, 1990) coding sequences were cotransformed into the *E. coli* strain BL21 along with the thermally inducible T7 polymerase plasmid, pGP1-2 (Tabor and Richardson, 1985). For rad6Δ_{153} and rad6Δ_{153} D120 V123, cells were grown at 30°C in LB (1% bactotryptone, 0.5% yeast extract and 0.5% NaCl) liquid media (containing 50 µg/ml ampicillin and 40 µg/ml kanamycin) to an absorbance of 0.4 at 590 nm. Cells were shifted to 42°C for one hour and then 37°C for two hours. Cells were then harvested by centrifugation, resuspended in 250 µl of 25% sucrose, 50 mM Tris-HCl (pH 8.0) and lysed with lysozyme as previously described (Gonda *et al.*, 1989). The cell lysates were centrifuged at 14 000 rpm for one hour after which protease inhibitors were added (antipain, aprotinin, chymostatin, leupeptin, pepstatin A (10 µg/ml) and phenylmethylsulfonyl fluoride (100 µg/ml) (Sigma)). The clarified supernatants were passed over a Mono Q HR 5/10 ion exchange column (Pharmacia) equilibrated with 50 mM

Tris-HCl (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM dithiothreitol (DTT) and the proteins were eluted with a NaCl gradient from 0 to 1 M. rad6Δ_{153} eluted at approximately 240 mM NaCl. The Mono Q fractions were concentrated using a centricon (Amicon) and then loaded onto a Superdex 75 HR 10/30 size exclusion column (Pharmacia) equilibrated with 50 mM Hepes (pH 7.5), 150 mM NaCl, and 1 mM EDTA. Glycerol (final concentration 5%) was added to the collected fractions and the protein samples were stored at -80°C.

Overexpression and preparation of a crude lysate of wheat E1 was carried out in a similar fashion as described above except for the following changes. Clarified cell lysates were dialyzed overnight at 4°C in 4 L of 10 mM Hepes (pH 7.5) and 1 mM DTT after which the sample was centrifuged at 17 000 rpm for 10 minutes. Protease inhibitors (as above) and glycerol (5%) were added to the supernatant which was then stored at -80°C.

Expression and radiolabeling of Ub was carried out as described previously (Ptak *et al.*, 1994). Cells were lysed with lysozyme using the procedure described by Gonda *et al.* (1989). Clarified supernatants were passed over a MonoQ HR 5/10 ion exchange column equilibrated with 50 mM Tris-HCl (pH 7.5), 1 mM EDTA and 1 mM DTT. The flow through was concentrated to 2 ml using a centricon and then run over a MonoS HR 5/10 ion exchange column (Pharmacia) equilibrated with 50 mM Hepes (pH 7.5), 1 mM EDTA and 1 mM DTT. The flow through was again collected and concentrated to 500 μl and then passed over a Superdex 75 HR 10/30 size exclusion column equilibrated with 50 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM EDTA and 1 mM DTT. Peak fractions were collected, pooled and concentrated to 500 μl. Glycerol was added to a final concentration of 5% and the protein samples were stored at -80°C. The concentration of Ub was determined using the BCA protein assay reagent (Pierce).

Detection of Ubiquitin-RAD6 Thiolesters

Reactions containing radiolabeled Ub (2µg, 200 000 cpm), wheat E1 (approximate final concentration of 4 nM) and E2 (4µg) were carried out in 500 µl of 10 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 40 mM NaCl, 5 mM adenosine triphosphate (ATP) supplemented with phenylmethylsulfonyl fluoride (180 µg/ml), chymostatin, pepstatin A, antipain, aprotinin and leupeptin (20 µg/ml), inorganic pyrophosphatase (0.6 units/ml) and an ATP regenerating system (2 µg/ml creatine kinase, 20 mM phosphocreatine). The ATP, protease inhibitors, inorganic pyrophosphatase and components of the ATP regenerating system were purchased from Sigma. Reactions were incubated for one hour at 30°C and then loaded onto a Superdex 75 16/60 size exclusion column equilibrated with 50 mM Hepes (pH 7.5), 150 mM NaCl and 1 mM EDTA. The number of counts in the Ub-E2 thiolester conjugate peak was determined.

The protein sequence alignments were obtained using the GCG programs for Multiple Sequence Analysis. The E2 images were reconstructed from the crystallographic coordinates of *S. cerevisiae* UBC4 (Cook *et al.*, 1993) and *A. thaliana* UBC1 (Cook *et al.*, 1992) using the molecular modeling program Insight II (Biosym/MSI, San Diego).

RESULTS

To determine what residues are involved in E2 specificity an attempt was made to transpose the function of one E2 to a functionally different E2 by altering specific surface residues. This gain of function strategy has the advantage over one that looks for a loss of function in that residues will be identified that are responsible for specificity rather than activities common to all E2s. Two E2s were chosen from *S. cerevisiae*, UBC4 and RAD6. RAD6 is involved in DNA repair, DNA damage induced mutagenesis, sporulation, repression of retrotransposition, and degradation of substrates by the amino end rule pathway (reviewed by Lawrence, 1994). UBC4 and its homologue UBC5 are part of a major proteolytic pathway responsible for degrading a large portion of short-lived and abnormal proteins (Seufert and Jentsch, 1990). They are also required under stress conditions and sporulation, and are heat shock and cadmium inducible. The reasons for choosing these particular E2s are threefold: they perform nonoverlapping functions, their biological properties can be measured using simple plating experiments, and the three dimensional crystal structures of *S. cerevisiae* UBC4 (Cook *et al.*, 1993) and the *A. thaliana* RAD6 homologue (Cook *et al.*, 1992) have been solved, allowing specific determinants to be mapped in three dimensions. Thus RAD6 and UBC4 are ideally suited for an analysis that correlates their structural differences with differences in their functions.

Mutagenesis Strategy

Examination of the recently solved three dimensional structures of *S. cerevisiae* UBC4 (Cook *et al.*, 1993) and the *A. thaliana* RAD6 homologue (Cook *et al.*, 1992) reveals that the backbone structures of these two E2s are essentially superimposable. Therefore it seems reasonable to assume that E2 specificity resides in the side chains of the surface residues. E2s are small molecules that interact with many different factors including Ub pathway enzymes as well as trans-acting factors specific to individual E2s. As a result of their relatively small sizes and such potentially conserved structures, different E2s would be expected to use the same surface region in binding an enzyme common to all E2s. Likewise the assumption was made that regions involved in binding trans-acting factors would map to corresponding positions on the E2 surface.

In addition to studying the three dimensional structures, the E2 linear amino acid sequences were also considered. Figure 1 is a sequence alignment of the catalytic domains of several UBC4 and RAD6 homologues. The six UBC4 members are 65% identical while the six RAD6 members are 53% identical. UBC4 and RAD6 from *S. cerevisiae* are 37% identical. Residues conserved in both the RAD6 and UBC4 homologues are most likely responsible for protein folding or for binding factors common to all E2s, such as Ub or E1. Residues conserved amongst the homologues of only one E2 family are probably involved in the particular functions of that family; residues not conserved within a family of E2s are probably inessential. To select potential specificity determinants the assumption was made that regions involved in trans-acting factor interactions of functionally different E2s would map to corresponding positions; thus residues were selected that were conserved at a particular position in each family, but different between the families. Twenty-one residues fit the criteria (figure 1, boxed residues), and they are distributed over the surface of the E2. Positions where small hydrophobic residues were present in both families were not selected; this was because it seemed less probable that substitution of these residues would alter E2 function. Specificity of an E2 is likely determined by surface residues involved in protein-protein interactions, and in fact out of the twenty-one residues selected, eighteen in the *A. thaliana* RAD6 homologue and nineteen in UBC4 are clearly on the surface of the protein. Residues 24 and 145 in the RAD6 homologue and residue 11 in UBC4 are only partially exposed, while residues 148 in the RAD6 homologue and 147 in UBC4 are essentially buried. The high proportion of these residues found on the surface of the proteins lends credence to the hypothesis that they might be involved in E2 function.

To test whether the selected residues were indeed determinants of E2 function, high copy plasmids were created that expressed rad6 derivatives that had the RAD6 specific residues changed to those found at the same position in UBC4 (figure 1, asterisks mark the residues that were changed). *S. cerevisiae* RAD6, unlike other RAD6 homologues and UBC4, possesses a short tail beyond its conserved catalytic domain. However, only determinants found within the catalytic domain were of interest and so rad6Δ, which lacks the tail, was used in this study. rad6Δ is still competent in DNA repair and UV induced mutagenesis (Morrison *et al.*, 1988). The rad6Δ derivatives were tested for their ability to function like UBC4 and for the loss of DNA repair activity. In this way it was hoped that residues important for UBC4 and RAD6 functions would be discovered.

ubc4ubc5 and *rad6* null mutants have a number of mutually exclusive and readily measurable phenotypes that can be used to facilitate a search for functional determinants.

The fact that *ubc4ubc5* null mutants grow very slowly and are extremely sensitive to the amino acid analog canavanine was employed to test whether any of the rad6Δ derivatives could function as UBC4. On the other hand, *rad6* null mutants are extremely sensitive to UV, which allowed testing of the substitutions for an effect on RAD6 function.

Most of the rad6Δ derivatives did not complement the slow growth phenotype or canavanine sensitivity of the *ubc4ubc5* null mutant, and all except one retained DNA repair activity (table 1). Some of the rad6Δ derivatives were slightly sensitive to UV, but because such a massive dose of UV was used, the role of these residues in DNA repair is probably minor. Retention of DNA repair activity indicated that the overall structures were not perturbed by the changes. Also because RAD6 function was retained and UBC4 activity was not created in most of the derivatives, these changes had no obvious effect on the specificity of the protein.

Substitution of Two Residues in rad6Δ Confers UBC4 Function

Interestingly, rad6Δ F65 N82 was able to partially complement the *ubc4ubc5* null phenotype while still retaining RAD6 activity. rad6Δ F65 N82 allowed the *ubc4ubc5* null mutant to grow significantly faster on plates, and cells were better able to survive on the amino acid analog canavanine (table 1). In addition, rad6Δ F65 N82 was more active than either rad6Δ derivative with a single substitution at position 65 or 82. Complementation by rad6Δ F65 N82 was not simply the result of increased expression of this derivative compared with rad6Δ, because the proteins were present at similar levels in the cell (results not shown). Thus, although residues 65 and 82 are inessential for RAD6 mediated DNA repair, they appear to be important for UBC4 activity.

For the above studies the rad6Δ derivatives were all expressed from high copy plasmids, but at high copy, with a gain of function assay, a slight effect on the function of a protein might become magnified. Therefore, to determine the relative importance of residues F65 and N82 to UBC4 function, rad6Δ F65 N82 was tested for its ability to complement the *ubc4ubc5* null phenotype at lower concentrations. The derivatives were placed on low copy vectors and their growth rates measured. The F65 N82 substitutions reduced the doubling time of the *ubc4ubc5* null strain to 5.1 hours compared with 8.4 hours with rad6Δ. Either substitution alone had less of an effect on the growth rate as cells expressing rad6Δ F65 had a doubling time of 5.9 hours, while cells expressing rad6Δ N82 had a doubling time of 8.3 hours. *UBC4* transformed cells doubled every 3.9 hours. rad6Δ F65 N82 complemented at low copy, and therefore transposition of only two residues from UBC4 to rad6Δ was sufficient to convey considerable UBC4 function on rad6Δ.

Identification of Residues Involved in RAD6 Function

Although most of the changes in rad6Δ did not affect its ability to function in DNA repair, rad6Δ D120 V123 was extremely sensitive to UV (table 1). To test their individual contribution to RAD6 function, residues 120 and 123 were substituted individually in rad6Δ. A *rad6* null strain transformed with a vector expressing rad6Δ D120 or rad6Δ V123 was sensitive to UV (figure 2A); however alteration of residue 120 created the most dramatic effect (approximately ten fold reduction in survival). The reduction in DNA repair activity was not simply the result of the rad6Δ derivatives being unable to accept Ub from E1; rad6Δ D120 V123 was capable of forming a Ub thiolester conjugate (results not shown). These results suggest that residues 120 and 123 may interact with trans-acting factors specific to RAD6.

The Amino Termini of UBC4 and RAD6 Are Functionally Interchangeable

The amino terminus of RAD6 has previously been shown to be an important determinant of RAD6 function. Watkins *et al.* (1993) demonstrated that residues 1-9 of RAD6 were involved in DNA repair and were necessary for amino-end rule dependent protein degradation and sporulation. In addition Sullivan and Vierstra (1991) found that deletion of the first eight residues of RAD6 decreased the rate of thiolester formation, with ubiquitin, to 15% of the wild-type enzyme. It was wondered whether, like RAD6, the amino terminus of UBC4 was important to its activity. To test this the first 10 residues of RAD6 were exchanged with the first eight residues of UBC4. As can be seen in table 1, a rad6Δ derivative containing the amino terminus of UBC4 (rad6Δ+UBC4 N terminus) retained RAD6 function, but failed to function like UBC4. Further, when the RAD6 amino terminus was placed on UBC4 (ubc4+RAD6 N terminus) this derivative behaved like wild-type UBC4 (table 1). These results suggest that the amino terminus of UBC4 is not involved in UBC4 activity. However, another interpretation is that the amino termini of RAD6 and UBC4 are functionally interchangeable, at least with respect to the phenotypes tested. In support of this, a *rad6* null strain expressing rad6Δ+UBC4 N terminus was resistant to UV (table 1). Thus the amino terminus of UBC4 can substitute for that of RAD6 in DNA repair and appears to contradict the results of other groups concerning the importance of the RAD6 amino terminus.

The above results seem less surprising when the RAD6 and UBC4 amino termini are compared; there are some common determinants (figure 1). Referring to the RAD6 sequence for numbering, position seven is a basic residue in both E2 families, either lysine or arginine, and position eight is a conserved arginine in both E2s. Sullivan and Vierstra (1991) have previously demonstrated the importance of residues 6-8 for RAD6-Ub thiolester formation. Therefore the basic residues common to both RAD6 and UBC4 (residues 7 and 8) were substituted to examine whether they were responsible for the ability of the amino termini of RAD6 and UBC4 to be interchanged. The residues were substituted for glutamine which is of similar size to arginine and lysine, but is neutral in charge. As shown in figure 2B, changing R8 in rad6Δ greatly reduced its DNA repair activity as did changing its UBC4 counterpart (R6 in rad6Δ+UBC4 N termini). Thus R8 is important for RAD6 activity as identified by Sullivan and Vierstra (1991) (although they never mutated this residue individually). Also, the presence of an arginine in the same position in the UBC4 amino terminus explains why it can replace that of RAD6, at least with respect to UV resistance.

With the information obtained from residue substitutions made in rad6Δ, an attempt was made to create a UBC4 derivative that possessed RAD6 activity. Alteration of two residues in rad6Δ (F65 N82) caused it to function, at least partially, as UBC4. It was wondered whether changing the corresponding positions in UBC4 (63 and 80) to the RAD6 specific residues would cause it to function as RAD6. In addition the residues corresponding to 120 and 123 (118 and 121) were changed because they appeared to be important for RAD6 function. Finally, the RAD6 N terminus, although functionally interchangeable with the amino terminus of UBC4 in terms of DNA repair, has been shown to be involved in a number of RAD6 functions (Watkins *et al.*, 1993). Therefore the amino terminus of UBC4 was replaced with that of RAD6. UBC4 N63 Y80 S118 N121 + RAD6 N terminus did not complement the UV sensitivity phenotype of the *rad6* null mutant (table 1). Thus, alteration of residues 63, 80, 118, 121 and addition of the N termini of RAD6 was not sufficient to impart RAD6 DNA repair activity to UBC4. These results correlate with those already mentioned; residues 65 and 82 appear not to be involved in RAD6 DNA repair activity as rad6Δ F65 N82 was resistant to UV. As well, overexpression of UBC4 N63 Y80 S118 N121 + RAD6 N terminus complemented the slow growth phenotype of the *ubc4ubc5* null strain and canavanine sensitivity to nearly wild-type levels (table 1). Thus, although residues 63 and 80 (65 and 82) were previously identified as being important to UBC4 function, they are not essential. Residues 63 and 80 may be only minor determinants of UBC4 function as alteration of these residues has a negligible effect on UBC4 activity. Alternatively lowering the expression of UBC4 N63 Y80 S118 N121 + RAD6 N terminus may reveal that these changes do indeed affect UBC4 activity (not tested). Nevertheless, residues involved in UBC4 function have been identified, and these results highlight an advantage of looking for residues involved in E2 specificity by testing for a gain of function.

DISCUSSION

To identify the structural determinants responsible for E2 activity, the RAD6 catalytic domain was substituted in an effort to make it function like UBC4. The results presented demonstrate that transposition of only two residues in rad6Δ, to the corresponding residues in UBC4 (F65 N82), results in significant complementation of the *ubc4ubc5* null phenotype. By looking for a gain of function rather than a loss of function it is known that changes in rad6Δ that bestow UBC4 activity are not perturbing E2 protein folding. Other residues important to UBC4 activity remain to be determined as rad6Δ F65 N82 does not possess wild-type UBC4 activity and changing the corresponding residues in UBC4 does not abolish its activity. One explanation for the latter result is that RAD6 possesses residues in common with UBC4 that when combined with F65 and N82 reproduce a UBC4 functional determinant. These residues would have to be altered in UBC4 along with F63 and N80 in order to eliminate UBC4 function. However these results do suggest that E2 activity may be determined by only a small number of surface residues.

In this study it has been found that residues 120 and 123 are probably important for specific functions of RAD6, since alteration of these residues in rad6Δ eliminates DNA repair activity without affecting E1 or Ub interactions. It is unlikely that changing these residues causes the protein to be misfolded for the following reasons: 1) residues 120 and 123 lie on the surface of the protein, 2) rad6Δ D120 V123 and rad6Δ elute with a similar profile during column purification, 3) substitution of the corresponding residues in UBC4 did not affect its activity, and 4) rad6Δ D120 V123 forms a Ub thiolester conjugate. It is also unlikely that the rate of thiolester formation with Ub is reduced, as initial studies suggest that rad6Δ and rad6Δ D120 catalyze this reaction at equivalent rates (results not shown). Therefore residues 120 and 123 are probably required because they interact with RAD6 substrates or trans-acting factors that are specific to RAD6.

A number of reasons probably contributed to the inability to discover more residues involved in E2 functions. First, in selecting residues to substitute, the assumption was made that regions involved in binding trans-acting factors would map to corresponding positions on the UBC4 and RAD6 surfaces. This hypothesis is obviously not completely correct because residues involved in the DNA repair function of RAD6 (120 and 123) are not involved in UBC4 activity. As well, if the hypothesis was correct more than two residues should probably have been discovered that could reconstruct UBC4 activity. Thus, it can be concluded that residues important for the specific functions of UBC4 and RAD6 are not necessarily found in the same position. Another reason that UBC4 wild-type activity was not reconstructed might be related to the way the substituted residues were clustered. In order to minimize the number of derivatives that had to be made, residues close to each other in the linear sequence were substituted in groups. A functional determinant in fact may be a combination of a number of residues that are not necessarily proximal in the linear sequence. Thus, functional determinants may not have been comprehensively reconstituted. Finally, more rad6Δ derivatives might have functioned as UBC4 if they had not retained their RAD6 activity. Presumably the rad6Δ derivatives still form complexes with trans-acting factors specific to RAD6, which may titrate out the amount of these derivatives available for UBC4 functions. Furthermore, while the intracellular location of UBC4 is unknown (although its substrates suggest it is found in the cytoplasm as well as the nucleus), RAD6 is concentrated in the nucleus (Watkins *et al.*, 1993). RAD6 forms a stable complex with RAD18 (Bailly *et al.*, 1994) which, like RAD6, is involved in DNA repair (Prakash, 1981). RAD18 also binds single stranded DNA and may sequester RAD6 to sites of DNA damage. This would presumably limit the amount of rad6Δ derivatives in the cytoplasm. Thus, small amounts of UBC4 activity may have been lost in the rad6Δ derivatives through their sequestration in the nucleus. It is therefore reasonable to say that a pragmatic solution to the problem of E2 specificity is more complicated that originally envisioned.

Previous work has suggested a role for the RAD6 amino terminus in both RAD6 specific functions and functions common to all E2s. Watkins *et al.* (1993) demonstrate that the amino terminus of RAD6 (residues 1-9) is necessary for efficient DNA repair and is essential for sporulation, degradation of substrates by the amino end rule pathway, and interaction with UBR1. McDonough *et al.* (1995) obtain similar results using charge to alanine scanning mutagenesis. In both studies the rad6 amino terminal derivatives are shown to form Ub thiolesters, but the authors do not test the rate at which this occurs. Sullivan and Vierstra (1991) however, find that two basic residues (7 and 8) at the amino terminus of the wheat RAD6 homologue are required for efficient thiolester formation with Ub. A large reduction in the rate of RAD6-Ub thiolester formation may partially account for the effect on RAD6 functions observed by the other two groups. However, the role of the RAD6 amino terminus is complicated; Watkins *et al.* (1993) find that UV induced mutagenesis is unaffected by its deletion and in fact is increased. This can be explained if amino end rule pathway degradation and sporulation, which are completely dependent on an intact RAD6 amino terminus, require a faster rate of Ub-RAD6 thiolester formation than either DNA repair or UV mutagenesis. Alternatively, the amino terminus of RAD6 may be bifunctional and be required for RAD6 specificity as well as Ub thiolester formation.

In light of the previous reports that point to the importance of the amino terminus in RAD6 function, it is interesting that the RAD6 amino terminus can be replaced by the amino terminus of UBC4; the derivative rad6Δ+ UBC4 N terminus is still active in DNA repair. Comparison of the amino termini of RAD6 and UBC4 reveals the presence of two common basic residues (7 and 8 in the RAD6 sequence). Changing this second residue in rad6Δ + UBC4 N terminus and rad6Δ totally eliminates DNA repair activity. Thus it appears that the UBC4 amino terminus can replace that of RAD6 because of a common correctly positioned basic residue. This arginine residue is conserved in all *S. cerevisiae* E2s for which the sequence is available. Thus, it is probable that this residue is required for some common E2 function, such as E1 or Ub binding, which is consistent with the results of Sullivan and Vierstra (1991). However, as the amino termini of all RAD6 homologues are almost invariant, it is expected that other residues in this region are responsible for bestowing full RAD6 activity, or are involved in RAD6 functions that have not been measured.

E2s interact with a number of different factors; some are common to all E2s such as Ub or E1 while others are specific to an E2. To make some predictions about what regions of an E2 may participate in conserved interactions, the amino acid sequences of ten *S. cerevisiae* E2s (UBC1-10) were compared using a program that aligned their sequences and assigned a number to each position that qualitatively defined how conserved the residues were at that position. Those residues that were highly conserved in all E2s were mapped onto the UBC4 and *A. thaliana* RAD6 structures. Also the sequences of known UBC4 and RAD6 homologues were compared, and the residues for each E[illegible]ere grouped according to degrees of conservation; the grouped residues were then mapped onto the respective E2 structures. Figures 3a and 3b show different views of UBC4 and *A. thaliana* RAD6, respectively, rotated by 90° in each panel. Two striking conserved grooves can be seen on the E2 surface, one above the active site and one to the right of it (blue). The groove above the active site continues as a highly conserved swath across the top of the E2 surface. It is reasonable to suggest that these regions are involved in Ub or E1 binding. The conserved grooves and the active site cysteine are surrounded, in the respective molecules, by residues that are specific to RAD6 and UBC4; this however is more striking in UBC4. Also visible is the highly conserved amino terminal region of RAD6, at the top of the molecule, that has been shown by a number of researchers to be important for RAD6 activity (Sullivan and Vierstra, 1991; Watkins *et al.*, 1993; McDonough *et al.*, 1995). On the sides of the molecules distal to the active sites long strips are formed by residues that are specific to UBC4 or RAD6. Overall it can be concluded that the patchworks, created by residues potentially required for common E2 functions (blue) and functions specific to UBC4 or RAD6 (red), are very similar for UBC4 and RAD6.

In addition to the conserved three dimensional maps presented in figure 3, it was also determined where residues that were shown empirically to be important to specific UBC4 and RAD6 functions lie on the surface of the molecules. Figure 4 shows that Phe 63 in UBC4 is close to the amino terminus of the protein directly above the active site; Asn 80 is situated beside the active site. The corresponding residues in RAD6 (Asn 65 and Tyr 82) occupy similar locations (not shown). Interestingly, a residue adjacent to Phe 63, Lys 64, has been previously identified as being necessary for the intermolecular monoubiquitination of UBC4 by itself *in vivo* (Gwozd *et al.*, 1995). Lys 64 is important for either the interaction of UBC4 with another UBC4 monomer or with a trans-acting factor required for UBC4 monoubiquitination. Also in this region is a proline residue (position 62 in UBC4) which is conserved in all E2s and is important for the cell cycle function of CDC34 (Goebl *et al.*, 1988) and the cell proliferation function of RAD6 (Ellison *et al.*, 1991). The conservation of this residue in all E2s argues against it being directly involved in interactions specific to an E2. However, this proline residue is part of the conserved E2 motif $PX_1X_2(P/S/A)P$, where X_1 is Phe 63 and X_2 is Lys 64 in UBC4. Proline, because of its rigid conformation, decreases the structural flexibility of the protein at that location. Thus, the proline residues in the motif $PX_1X_2(P/S/A)P$ probably function to hold the residues X_1X_2 in the proper orientation needed to be recognized by specific trans-acting factors. It is reasonable to speculate that this region is important for the specific functions of other E2s in addition to UBC4. Although changing X_1 in RAD6 (Asn 65) did not eliminate the DNA repair activity of RAD6, it is possible that this region is necessary for other RAD6 functions or is part of a larger determinant that remains to be uncovered.

The amino terminal arginine conserved in all E2s (R6 and R8 in UBC4 and RAD6 respectively) is located at the top of either molecule (figures 4 and 5). It was determined that the amino terminus of UBC4 could substitute for the RAD6 amino terminus because of this common residue. The arginine side chains are in different positions in the crystal structures of UBC4 and RAD6, but are flexible based on the crystallographic data.

Figure 5 shows that residues Ser 120 and Asn 123 in RAD6, identified in this study as being necessary for DNA repair, lie beside the active site with Ser 120 directly below it.

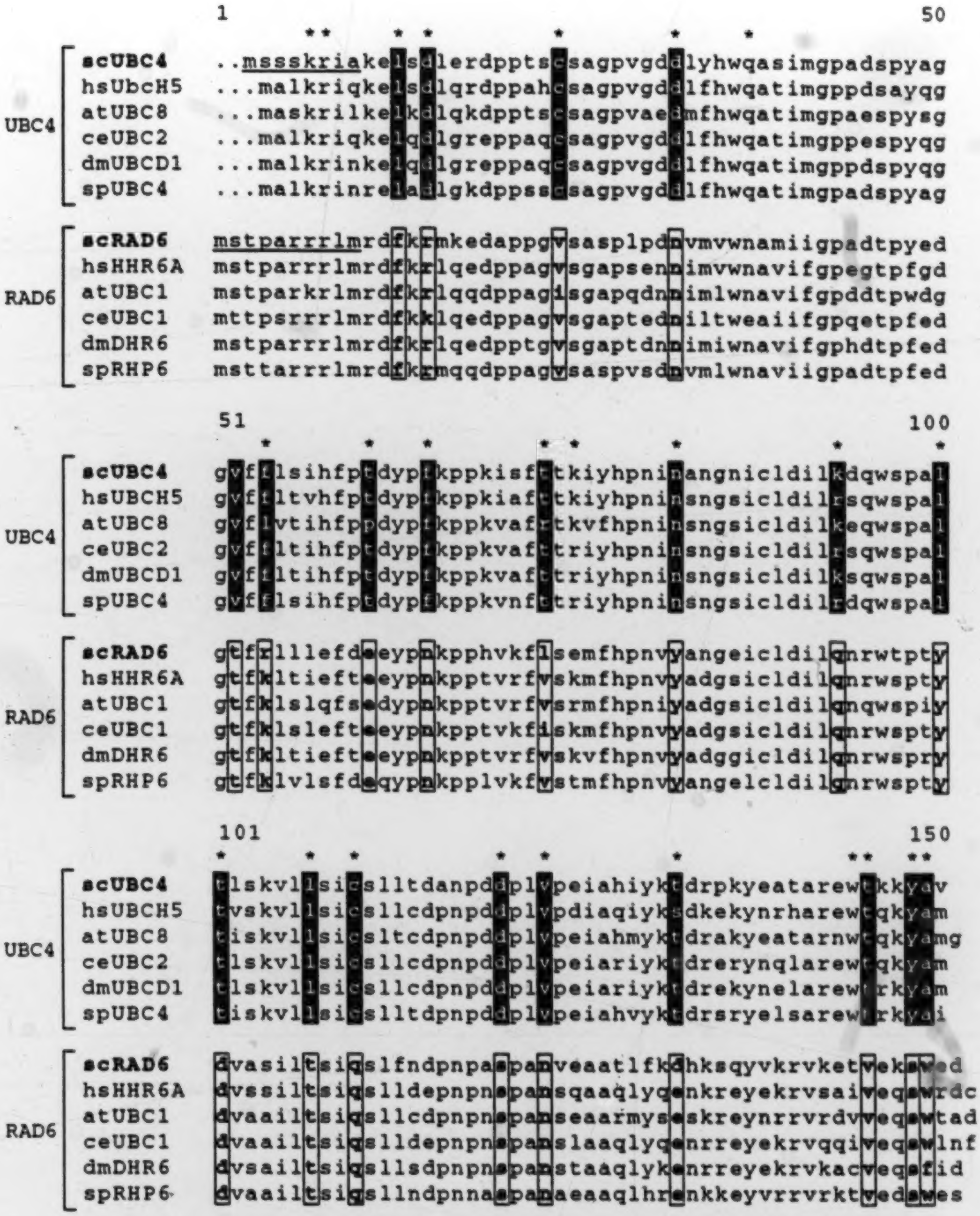
CDC34 also has a serine residue at this position; in contrast UBC4 and a number of UBC1-like E2s have an aspartate residue. Liu *et al.*, (1995) changed this serine residue (Ser 139) in CDC34 to an aspartate and found that this derivative was able to complement a *cdc34* temperature sensitive or disruption strain only at high copy, but not at low copy. Thus, like RAD6, this residue appears to be important for CDC34 function. It is interesting that the common serine residue is found in an area that is highly conserved between RAD6 and CDC34, but not other E2s, which suggests that this region is important for trans-acting factor interactions in these E2s. That RAD6 and CDC34 have some similar functional determinants is supported by the observation that a chimeric protein, composed of the RAD6 catalytic domain fused to the CDC34 tail, possesses wild-type CDC34 cell cycle activity (Kolman *et al.*, 1992; Silver *et al.*, 1992).

Other residues that are clearly relevant to the emerging picture of E2 specificity have been reported and are worthy of mention. McDonough *et al.* (1995), using charge to alanine scanning mutagenesis, find that residues 49, 50, 71, 75, 86 and 90 are important for DNA repair. Residues 49, 50, 86 and 90 are also essential for sporulation and N end rule degradation (71 and 75 were not tested). Residues 71 and 75 were substituted together, however the present work demonstrates that changing residue 75 in the RAD6 catalytic domain does not result in a significant loss of DNA repair activity; therefore residue 71 is probably the functional determinant. Residues 71, 86 and 90, along with 120 and 123, are congregated around the active site (figure 5) and appear to define a region involved in RAD6 specific functions. Residues 49 and 50 are present on the surface distal to the active site (figure 5).

Although this discussion has focused on functional determinants that are common in homologues of an E2, information about individual isoforms of an E2 family can also contribute to the picture of specificity. Three UBC4 isoforms have been identified in rat (Wing and Jain, 1995; Wing *et al.*, 1996). One isoform, UBC8A, is expressed only in the testis and is developmentally regulated, being induced during puberty (Wing *et al.*, 1996).

The three isoforms differ in their ability to conjugate Ub to a fraction of testis proteins, and this difference in function is due to four residue substitutions (16, 50, 108 and 126, *S. cerevisiae* UBC4 numbering; S. Wing, personal communication). Residues 16, 50, and 108 are conserved in all UBC4 homologues except rat UBC8A. Residue 126 is either a histidine or an arginine in all UBC4 homologues except human UbcH5A, *S. cerevisiae* UBC5, and rat UBC8A where it is a glutamine. Residues 16, 50, and 108 lie on the backside of the molecule, relative to the active site (figure 6). This face is highly conserved in UBC4 homologues (figure 3a). Substitution of these residues either allows UBC8A to interact with a distinct set of proteins, for example substrates or E3s, or inhibits UBC8A from interacting with factors common to the other UBC4 isoforms. It will be interesting to see if there are other UBC4 isoforms, yet to be identified, that use the same surface of the molecule for interacting with distinct trans-acting factors. In conclusion, from the combined studies of several research groups, the regions involved in E2 specificity are slowly being delineated.

Clearly several of the derivatives described here have potential use in biochemical or genetic screens designed to isolate trans-acting factors involved in E2 specificity. By this it is hoped that a better understanding of how E2s function can be gained.


1
50
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100
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Figure 1: Sequence comparison of UBC4 homologues with RAD6 homologues. Boxes indicate residues that are conserved in all UBC4 homologues (filled) or in all RAD6 homologues (open) but are different between the two groups. (Note the *Arabidopsis* E2, UBC8 was not included in the original sequence alignment and so several residueswere selected that were not conserved in this sequence.) The following are considered conservative changes: R and K, V I and L, S and T, E and D. Asterisks mark the residues that were mutated. The amino terminal sequences that were exchanged are underlined. Abbreviations: sc, *Saccharomyces cerevisiae*; hs, *Homo sapien*; at, *Arabidopsis thaliana*; ce, *Caenorhabditis elegans*; dm, *Drosophila melanogaster*; sp, *Schizosaccharomyces pombe*.

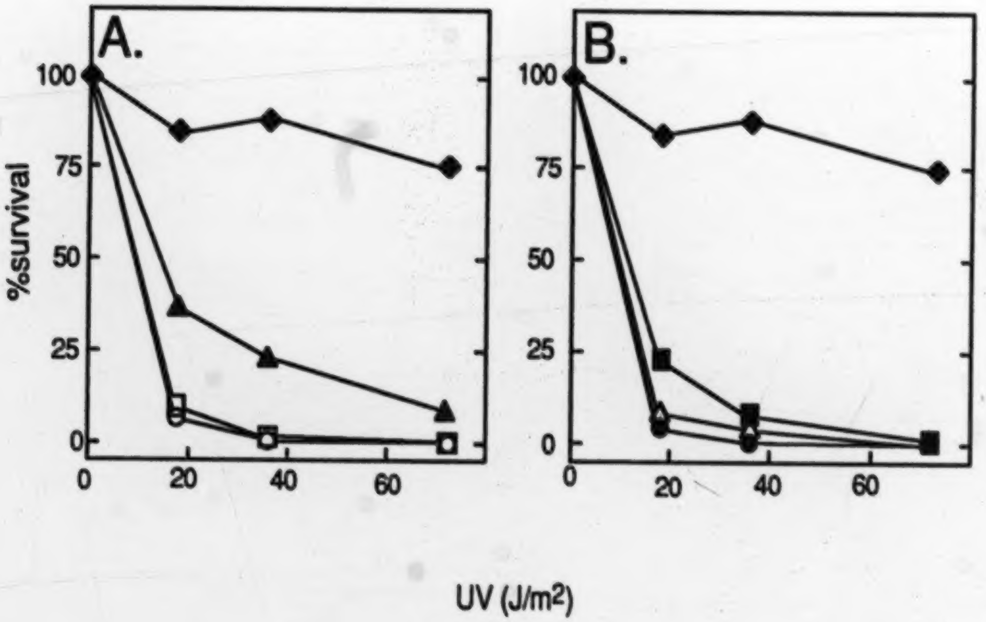

Figure 2: UV resistance of rad6Δ mutants in a *rad6* null strain. % survival is the number of colonies formed after UV irradiation compared with an unirradiated control. ◆ rad6Δ, □ null (plasmid lacking RAD6), ▲rad6Δ V123, ○rad6Δ D120, ■ rad6Δ Q8, △rad6Δ + UBC4 NT Q6, ●rad6Δ + UBC4 NT Q5,6. NT- amino terminus.

Figure 3a

Three dimensional space-filling images of *S. cerevisiae* UBC4 rotated by 90°. The active site cysteine is colored yellow. Residues that are highly conserved in all ubiquitin conjugating enzymes are colored blue. All other residues are colored from white to red; the darker the shade of red, the more highly conserved the residue is among the UBC4 homologues. The images were reconstructed from the crystallographic coordinates (Cook *et al.*, 1993) using the molecular modeling package Insight II (Biosym/MSI, San Diego).

Figure 3b

Three dimensional space-filling images of the *A. thaliana* RAD6 homologue rotated by 90°. The active site cysteine is colored yellow. Residues that are highly conserved in all ubiquitin conjugating enzymes are colored blue. All other residues are colored from white to red; the darker the shade of red, the more highly conserved the residue is among the RAD6 homologues. The images were reconstructed from the crystallographic coordinates (Cook *et al.*, 1992) using the molecular modeling package Insight II (Biosym/MSI, San Diego).

Figure 4

Three dimensional space-filling image of *S. cerevisiae* UBC4 showing the positions of the active site cysteine (C86), the amino terminal arginine common to UBC4 and RAD6 (R6), UBC4 functional determinants (F63 and N80), and the lysine affecting UBC4 ubiquitination (K64). See figure legend 3a for the significance of other colored residues. The image was reconstructed from the crystallographic coordinates of UBC4 (Cook *et al.*, 1993) using the molecular modeling package Insight II (Biosym/MSI, San Diego).

Figure 5

Three dimensional space-filling images of the *A. thaliana* RAD6 homologue, rotated by 180°, showing the positions of the active site cysteine (C88), the amino terminal arginine common to RAD6 and UBC4 (R8), RAD6 functional determinants (S120 and N123), and residues substituted by McDonough *et al.* (1995) that affect RAD6 function (D49, G50, R71, S86, and D90). See figure legend 3b for the significance of other colored residues. The images were reconstructed from the crystallographic coordinates (Cook *et al.*, 1992) using the molecular modeling package Insight II (Biosym/MSI, San Diego).

Figure 6

Three dimensional space-filling images of *S. cerevisiae* UBC4, rotated by 180°, showing residues responsible for the distinct specificities of two rat UBC4 isoforms (16, 50, 108, and 126, *S. cerevisiae* numbering; S. Wing, personal communication). See figure legend 4 for the significance of the other colored residues. The images were reconstructed from the crystallographic coordinates of UBC4 (Cook *et al.*, 1993) using the molecular modeling package Insight II (Biosym/MSI, San Diego).

Table 1 Phenotypes of rad6Δ and ubc4 Derivatives

E2[a]	Changes	Growth at 30°C [b]	Canavanine Resistance (%)[c]	UV Resistance (%)[d]
rad6Δ [e]		+	-	93
UBC4		++++++	33	0
null [f]		+	-	0
rad6Δ	F65	+++	4	71
rad6Δ	N82	++	0.2	98
rad6Δ	F65 N82	++++	13	47
rad6Δ	D120 V123	+	-	0
rad6Δ	UBC4 NT [g]	+	-	70
UBC4	RAD6 NT	++++++	42	0
UBC4	RAD6 NT N63 Y80 S118 N121	+++++	25	0
rad6Δ	L13 D15 C24	+	-	94
rad6Δ	D32 Q37	++	-	75
rad6Δ [h]	F54 T73 K75 W144 T145 Y148 A149	+	-	58
rad6Δ	T61	+	-	83
rad6Δ	K93	+	-	91
rad6Δ	L100 T101 L107 C110	+	-	39
rad6Δ	T132	+	-	90

[a] expressed from high copy plasmids

[b] mutants transformed into the *ubc4ubc5* null strain were grown on SD media containing the required amino acids. Growth was scored based on colony size. Colonies were given a designation from + to ++++++ based on their relative sizes compared with colonies expressing rad6Δ or UBC4 respectively.

[c] mutants transformed into the *ubc4ubc5* null strain were grown on SD media containing the required amino acids plus 1.5 μg/ml canavanine. Resistance was calculated as the number of colonies formed after 7 days on SD plus canavanine plates relative to the number of colonies on SD plates alone. '-', if in an initial experiment no significant canavanine resistance was bestowed to the *ubc4ubc5* null mutant, further analysis was not done.

[d] mutants transformed into the *rad6* null strain were irradiated with 72 J/m^2 UV. Resistance was calculated as the number of colonies formed after UV irradiation relative to an unirradiated control.

[e] rad6Δ - rad6 catalytic domain (rad6Δ_{153})

[f] control plasmid lacking an insert

[g] NT - amino terminus

[h] rad6Δ_{150}, all other derivatives are rad6Δ_{153}

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Chapter IV

p53 Is Stable in Yeast Even in the Presence of HPV-16 E6 and E6AP

INTRODUCTION

The tumor suppressor protein p53 is commonly found inactivated in a wide variety of human cancers (Hollstein *et al.*, 1991; Levine *et al.*, 1991). In normally functioning cells, DNA damage causes cells to arrest in G1 of the cell cycle or to be destroyed through apoptosis. p53 is involved in these processes and probably through its activity as a sequence specific DNA binding transcription factor increases the expression of target genes whose products are necessary to block cell proliferation or cause cell death. Cells in which p53 has been inactivated are able to continue through the cell cycle, but fail to repair DNA lesions that can lead to mutations in the genome and possibly tumorigenesis. Inactivation of p53 can occur either by mutation (reviewed by Soussi and May, 1996), viral oncoproteins (Lane and Crawford, 1979; Linzer and Levine, 1979; Sarnow *et al.*, 1982; Zantema *et al.*, 1985), or the cellular protein mdm2, a negative regulator of p53 function (Wu *et al.*, 1993).

The human papilloma virus (HPV) is one culprit involved in p53 inactivation. HPVs that infect the anogenital tract are divided into high and low risk depending on the frequency with which they are found associated with cancer. High risk viruses (HPV-16 and -18) are found in approximately 90% of cervical cancers (zur Hausen and Schneider, 1987; Riou *et al.*, 1990); low risk viruses (HPV-6 and -11) are associated with benign lesions. HPV encodes two oncoproteins E6 and E7 that cause immortalization of primary human squamous epithelial cells (Münger *et al.*, 1989; Hawley-Nelson *et al.*, 1989). E7 has been shown to bind to the retinoblastoma protein (Dyson *et al.*, 1989), while E6 associates with p53 (Werness *et al.*, 1990). HPV positive carcinomas contain wild-type p53, but the half life of p53 is reduced along with the steady state levels of the protein (Scheffner *et al.*, 1991). E6 forms a complex with the cellular protein E6 associated protein (E6AP), and together they interact with p53 (Huibregtse *et al.*, 1991). E6 and E6AP appear to function as an E3 that targets p53 for rapid Ub dependent degradation (Scheffner *et al.*, 1993).

The majority of work aimed at dissecting p53 degradation has been carried out *in vitro* using either *in vitro* translated or recombinant proteins. *S. cerevisiae* represents a genetically tractable system for the study of p53 degradation. Thus far a p53 homologue has not been isolated from yeast; however yeast cells contain the evolutionary conserved Ub system shown to be responsible for p53 proteolysis. An attempt was made to reconstitute p53 degradation *in vivo* with the intent of then being able to address a number of questions about this process. Specifically, although homologues of *S. cerevisiae* UBC4 have been shown to ubiquitinate p53 *in vitro* (Scheffner *et al.*, 1994), it needs to be demonstrated that UBC4 is responsible for this activity *in vivo*. Another E2 that appears to be unrelated to the UBC4 family of E2s is also able to ubiquitinate p53 *in vitro* (Ciechanover *et al.*, 1994; Nuber *et al.*, 1996). Whether there are additional E2s with this activity could be determined using yeast E2 null strains. As well, the *in vivo* site of p53 ubiquitination could be mapped by introducing *p53* mutants into yeast. Ultimately a simple *in vivo* system would be of great value in examining ways to inhibit E6 mediated p53 degradation. If p53 could be stabilized this might prevent the development of cancer in patients infected with high risk HPVs.

In this study *S. cerevisiae* was used as a model system to reconstruct p53 degradation.

MATERIALS AND METHODS

Plasmids and Yeast Strains

Vectors php53, pGEME6AP and pGEM16E6 were kindly provided by P.M. Howley and contain the wild-type human *p53* and *E6AP* sequences and the HPV *E6* sequence respectively. The Altered Sites II *in vitro* Mutagenesis System (Promega), for *p53* and *E6AP*, and the polymerase chain reaction (PCR), for *E6*, were used to introduce a *Sst* I site at the 5' terminus and a *Kpn* I site at the 3' terminus of the genes. The introduction of these sites allowed the subsequent transfer of the gene sequences into yeast expression vectors. The yeast high copy *TRP1* and *LEU2* p53 expression vectors are identical, respectively, to YEp96 (Ellison and Hochstrasser, 1991) and the *LEU2* vector previously described by Silver *et al.* (1992) except that the human *p53* gene sequence replaces the *Ub* and the *UBC4* coding sequences. The yeast high copy *TRP1* hemagglutinin (HA) tagged E6AP (hE6AP) and E6 (hE6) expression vectors are identical to that described for amino terminally HA tagged UBC4 (hUBC4, Gwozd *et al.*, 1995) except that the *E6AP* and *E6* gene sequences replace that of *UBC4*. The yeast high copy *URA3* hE6AP expression plasmid is identical to the *URA3* vector previously described by Silver *et al.* (1992) except the *hE6AP* sequence (encoding residues 213 to 865, 76 kDa form) replaces that of *CDC34*. The HA tag is the same as that described for hUBC4 (Gwozd *et al.*, 1995). An *Sst* I site was created at the 5' end of the *p53* and *E6* sequences by incorporating two serine codons after the methionine start codon (ATG **AGC TCA**, *Sst* I site in bold). A *Sst* I site was created at the 5' end of the E6AP sequence by adding three codons 5' of codon 213 (ATG **AGC TCA**, *Sst* I site in bold). The coding sequence of HPV *E6* was verified by DNA sequencing using an Applied Biosystems 373A DNA sequencer operated by the University of Alberta, Department of Biochemistry, DNA Synthesis and Sequencing Laboratory.

The high copy *TRP1* negative control plasmid pES12 (Hodgins *et al.*, 1992) is identical to YEp96 except for deletion of the *Ub* coding sequence. The high copy *URA3* negative control plasmid YEp352 has been previously described (Hill *et al.*, 1986).

The high copy plasmids were introduced either singly or in combination into the yeast strain MHY501 (obtained from M. Hochstrasser). MHY501 is a wild-type strain and has the genotype: *MATα his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1*.

In Vivo Labeling and Immunoprecipitation

To detect expression of p53 and hE6AP, cells harboring either a *TRP1* high copy expression vector or a control vector were grown overnight at 30 °C in semisynthetic media (0.15% yeast extract, 0.1% KH_2PO4, 0.1% NH_4Cl, 0.04% $CaCl_2$, 0.05% NaCl, 0.06% $MgCl_2$-$6H_20$, 2% glucose) plus lysine (40 mg/L), leucine (60 mg/L), histidine (10 mg/L) and uracil (40 mg/L). Cells were diluted into early exponential phase and allowed to go through two doublings in the presence of 100 μM $CuSO_4$. Cells were pelleted and resuspended to an OD_{600}=10 in prewarmed labelling buffer (40 mM potassium phosphate buffer pH 6.0, 2% glucose, required amino acids, 100 μM $CuSO_4$, 30°C). Cells were labelled with *trans*-[^{35}S]-methionine (80 μCi/ml of resuspended cell culture) (ICN) for five minutes at 30°C with vigorous shaking. 0.5 ml aliquots were taken, added to 75 μl of fresh 1.85M NaOH and 7.4% β-mercaptoethanol and incubated on ice for 10 minutes. 60 μl of 55% trichloroacetic acid (TCA) was added and the samples were again incubated on ice for 10 minutes. Samples were centrifuged for 10 minutes at 14 000 rpm and the pellets were washed in 1 ml ice cold acetone. The samples were resuspended in 115 μl of 2% SDS, 100 mM Tris-HCl pH 7.5 and heated to between 65 and 90°C for 10 minutes. Samples were centrifuged and the supernatants were kept. 45 μl of the 2% SDS samples were added to tubes containing 1 ml of BTNTE (2 mg/ml BSA, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA and 0.02% NaN_3), phenylmethylsulfonyl fluoride (1 mM) (SIGMA), leupeptin, antipain, chymostatin, aprotinin and pepstatin A (10 μg/ml) (SIGMA). Either monoclonal α-HA antibody (12CA5 from T. Hobman) or 5 μl of α-p53 monoclonal antibody (SIGMA) were added and the solutions were rocked at 4°C overnight. Samples were centrifuged for 10 minutes and then the supernatants were removed to new tubes containing 16 μl of a 50% protein-A-sepharose (SIGMA) slurry in BTNTE. The samples were incubated for 1 hour at room temperature with rotation. Next the samples were spun briefly and the supernatants were removed. 1 ml of NaTNTE was added (50 mM Tris-HCl pH 7.5, 1 M NaCl, 1% Triton X-100, 5 mM EDTA) and the samples were incubated for 5-10 minutes with rotation. The samples were centrifuged and the supernatants removed. The beads were washed with 1 ml of NaTNTE. Incubations and washes were repeated with TNTE (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA) and then NTE (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA). The buffer was removed, 20 μl of IPLB was added (0.1 M Tris-HCl pH 6.8, 5% SDS, 5 mM EDTA, 0.005% bromophenol blue, 25% glycerol, 5% β-mercaptoethanol) and the samples were heated for 10 minutes at 65-90 °C. Samples were electrophoresed on a sodium dodecyl sulfate (SDS) polyacrylamide gel (18% acrylamide, 0.09% bisacrylamide) and autoradiography was performed.

To detect expression of hE6, cells harboring either a *TRP1* high copy expression vector or a control vector were grown at 30 °C in synthetic defined (SD) media (Sherman *et al.*, 1986) supplemented with lysine (40 mg/L), leucine (60 mg/L), histidine (10 mg/L) and uracil (40 mg/L). Cells in late exponential phase were pelleted followed by resuspension in electrophoresis load mix (12.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 0.001% bromophenol blue, 200 mM dithiothreitol (DTT)). Samples were then boiled for 10 minutes and centrifuged to remove cellular debris. Sample supernatants were electrophoresed on an SDS polyacrylamide gel (18% acrylamide, 0.09% bisacrylamide) and Western analysis was performed using a monoclonal α-HA antibody and the same procedure described by Gwozd *et al.* (1995).

For determination of p53 degradation in yeast, cells were grown overnight in semisynthetic media plus lysine (40 mg/L) and histidine (10 mg/L). Cells were diluted into early exponential phase and allowed to go through two doublings in 100 μM $CuSO_4$. The cultures were then pelleted and resuspended to an OD_{600}=10 in prewarmed labelling buffer. After labelling cells for 5 minutes with *trans*-[^{35}S]-methionine (80 μCi/ml of resuspended cell culture) (ICN), cells were pelleted and then resuspended to an OD_{600}=10 in semisynthetic media containing lysine (40 mg/L) and histidine (10 mg/L), cycloheximide (final concentration of 1 mg/ml of resuspended cell culture), and cold methionine (20 mM). 0.5 ml aliquots were taken at 0, 30 and 60 minutes, added to fresh 75 μl of 1.85 M NaOH and 7.4% β-mercaptoethanol and incubated on ice for at least 10 minutes. 60 μl of 55% TCA was added and the samples were again incubated on ice for 10 minutes. Samples were centrifuged for 10 minutes at 14 000 rpm and the pellets were washed in 1ml ice cold acetone. The samples were resuspended in 115 μl of 2% SDS, 100 mM Tris-HCl pH 7.5

and heated to between 65 and 90°C for 10 minutes. Samples were centrifuged and the supernatants were kept. The number of counts per minute (cpm) in each sample was measured and volumes with equivalent amounts of cpm were added to tubes containing 1 ml of BTNTE, phenylmethylsulfonyl fluoride (1 mM) (SIGMA), leupeptin, antipain, chymostatin, aprotinin and pestatin A (10 μg/ml) (SIGMA). 1 μl of α-p53 monoclonal antibody (SIGMA) was added and the solution was rocked at 4°C overnight. The rest of the procedure was carried out as described above for detection of p53 and hE6AP expression.

RESULTS

The first step in this study was to express p53 in *S. cerevisiae*. p53 was placed on a yeast/*E. coli* shuttle vector behind the *CUP1* promoter which is $CuSO_4$ inducible and normally provides high levels of gene expression. Cells were metabolically labeled with trans-[^{35}S]-methionine and lysed, and α-p53 antibody was added to immunoprecipitate p53. The immunoprecipitated samples were analyzed by SDS-PAGE followed by autoradiography. As can be seen in figure 1A, a protein with a molecular weight of approximately 53 kDa was detected in yeast cells transformed with the p53 expression vector, but not in cells harboring a control vector. Although p53 expression in *S. cerevisiae* has previously been shown to inhibit growth (Nigro *et al.*, 1992), this was not detected (results not shown). This could be explained by the relatively low levels of p53 that were present. High levels of p53 expression appear to be necessary to inhibit growth (Nigro *et al.*, 1992).

p53 Is Stable in *S. cerevisiae*

Before developing an *in vivo* system to study p53 degradation, it first had to be ensured that p53 alone was relatively stable in yeast. The metabolic stability of p53 was determined using a time course experiment in which cells were labeled briefly with trans-[^{35}S]-

methionine. At time zero cold methionine and cycloheximide were added. Samples were taken at the indicated times and lysed, and α-p53 antibody was added to immunoprecipitate p53. Figure 2 (lanes 1-3) shows that over 60 minutes p53 is quite stable. The stability of p53 in yeast contrasts with the relatively short half life of p53 in mammalian cells (Rogers *et al.*, 1986).

Next E6 and E6AP (75 kDa) were also expressed in yeast. The 75 kDa form of E6AP has all the defined properties of full length E6AP; it associates with E6, mediates binding of E6 to p53, and facilitates the ubiquitination of p53 in the presence of E6. To detect E6AP and E6, these proteins were tagged with the hemagglutinin (HA) epitope from influenza virus. The HA tagged versions (hE6AP and hE6) were placed on a yeast/*E. coli* shuttle vector behind the *CUP1* promoter. Cells transformed with the hE6AP or hE6 expression vectors or with a control vector, were labeled with trans-[^{35}S]-methionine, and α-HA antibody was added to the lysates to immunoprecipitate the HA tagged proteins. The samples were analyzed by SDS-PAGE and autoradiography. A protein of the expected molecular weight for hE6AP was detected in cells transformed with the hE6AP expression vector but not in cells harboring the control vector (figure 1b). For hE6 a protein of approximately 24 kDa was immunoprecipitated from cells expressing hE6 but not in the control cells (results not shown). This is about 3 kDa greater than the expected molecular weight of 20.9 kDa. The E6 expression vector was sequenced to ensure the coding sequence was correct. One possible explanation for the discrepancy in molecular weight is that hE6 is posttranslationally modified in yeast. However this is probably not the case as detection of hE6 by Western analysis with α-HA antibody reveals a protein of the correct molecular weight (figure 1C). The reason for the difference between the immunoprecipitation and Western results is not known. Nevertheless, hE6AP and hE6, which are necessary for p53 degradation *in vitro*, can be expressed in yeast.

Coexpression of E6 and E6AP Does Not Destabilize p53

With the ability to express all three proteins in yeast, it was next determined whether hE6AP and hE6 co-expression could shorten the half-life of p53. Yeast cells were transformed with three vectors; each contained a different selectable marker and expressed either p53, hE6AP, or hE6. A pulse-chase experiment similar to the one described above was carried out. p53 was stable in the presence of hE6AP and hE6 (figure 2, lanes 4 to 6). Furthermore higher molecular weight species that could possibly represent p53-Ub conjugates were not detected (results not shown). Thus although E6AP and E6 appear to be necessary and sufficient for p53 degradation *in vitro*, they do not direct p53 degradation in yeast.

p53, E6AP, and E6 have been shown to form a trinary complex *in vitro* (Huibregtse *et al.*, 1991). Isolation of this complex from yeast was attempted using immunoprecipitation and the α-HA antibody, but no evidence could be found for its formation (results not shown).

DISCUSSION

There are a number of possible reasons why p53 degradation could not be reproduced in yeast. First, factors other than E6 and E6AP may be required for efficient p53 degradation. In support of this idea is the observation that ubiquitination of p53 *in vitro* using purified and recombinant components is somewhat inefficient (Rolfe *et al.*, 1995). Furthermore, E6AP expressed in bacteria is less active in the ubiquitination of an E7-E6 fusion protein than a E6AP containing fraction from rabbit reticulocyte lysate (Scheffner *et al.*, 1993). Alternatively one of the components, either p53, E6AP, or E6, may have been improperly folded, or their activities may require posttranslational modifications provided only in mammalian cell systems. E6 has previously been expressed in yeast; however the majority of the protein produced was insoluble in cell lysates (Carter *et al.*, 1991). E6 inclusion body formation was not tested, but low levels of soluble E6 might account for the inability to degrade p53. Another reason for the stability of p53 may be that *S. cerevisiae* UBC4 is not capable of mediating ubiquitination of p53. Thus far only one human and one *A. thaliana* UBC4 homologue have been shown to mediate p53 ubiquitination (Scheffner *et al.*, 1994). *S. cerevisiae* and *A. thaliana* UBC4 possess approximately the same amount of sequence identity with the human UBC4 homologue (Jensen *et al.*, 1995), but the human and plant homologues do have a few residues in common that differ in the *S. cerevisiae* protein. These residues may be important for mediating p53 degradation. Further, a recently identified human E2, thought to be a member of the UBC4 family, was found to be inefficient in p53 ubiquitination (Nuber *et al.*, 1996). It has been demonstrated that a small number of residues can dramatically alter the function of an E2. The substitution of only two residues in RAD6 imparted UBC4 function to RAD6 (chapter 2). As well, a testis specific rat UBC4 homologue exists that differs from other rat UBC4 homologues in its ability to conjugate Ub to a fraction of testis proteins (Wing *et al.*, 1996), and this is the result of only four distinct residues (S. Wing, personal communication).

In conclusion, the inability to reconstitute Ub dependent degradation of p53 in yeast suggests that this problem is clearly more complicated than was originally presumed.

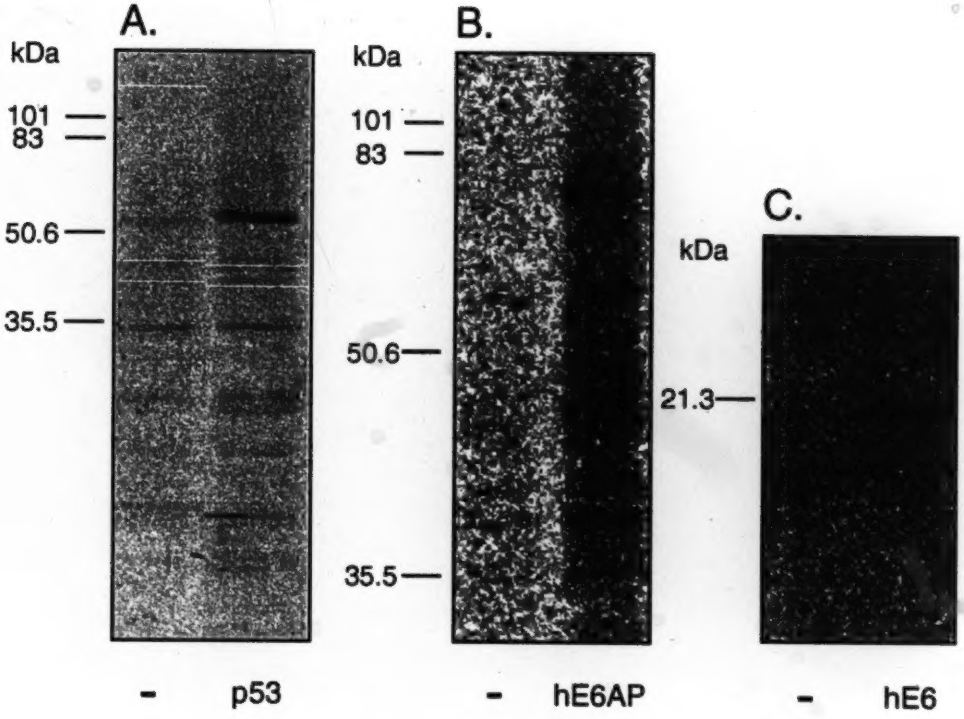

Figure 1: Expression of p53, hE6AP and hE6 in *S. cerevisiae*. In panels A and B, cells were metabolically labeled with trans-[^{35}S]-methionine and then lysates were immunoprecipitated with α-p53 or α-HA antibodies respectively. Immunoprecipitates were analyzed by SDS PAGE and autoradiography. In panel C, cells were lysed and Western analysis was performed using α-HA antibodies. The migration of molecular weight standards (kDa) are shown to the left of each figure. "-" refers to cells transformed with a plasmid lacking the appropriate insert.

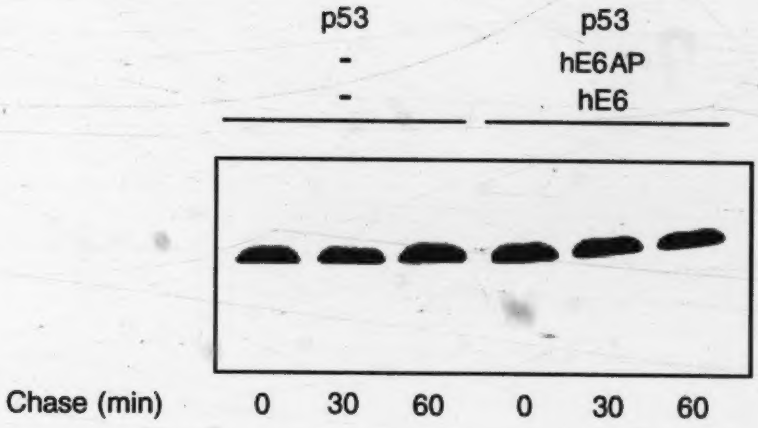

Figure 2: Metabolic stability of p53 in *S. cerevisiae*. Wild-type cells were cotransformed with plasmids expressing p53, hE6AP, or hE6, or control plasmids lacking an insert. Cells were briefly labeled with trans-[^{35}S]-methionine and then chased with cold methionine and cycloheximide. Aliquots were taken and lysed at the times indicated. Lysates were immunoprecipitated with α-p53 antibody. The immunoprecipitates were analyzed by SDS PAGE and autoradiography. "-" refers to plasmids lacking an insert.

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Chapter V

General Discussion

The focus of these studies has been to identify the structural requirements of specific E2 functions. E2s potentially interact with a number of different proteins, with some being specific to individual E2s. Identification of residues involved in E2 functions, and therefore transacting factor interactions, will hopefully lead to a better understanding of how E2s accomplish their various activities.

E2 Ubiquitination

UBC4 monoubiquitinates itself *in vivo*, and this can be eliminated by substitution of either the targeted lysine (K144) or a separate lysine (K64). The fact that substitution of K64 abolishes UBC4 ubiquitination indicates that this residue interacts with a transacting factor responsible for this activity; K64 may interact with either another UBC4 monomer or an E3. No phenotype is associated with UBC4 derivatives that are incapable of being ubiquitinated, so the functional significance of this finding is unclear. Other E2s have also been shown to be able to ubiquitinate themselves. CDC34 is ubiquitinated *in vitro* (Banerjee *et al.*, 1993) and *in vivo* (Goebl *et al.*, 1994). This reaction differs from UBC4 ubiquitination in that CDC34 is multiubiquitinated in an intramolecular reaction. Because the presence of a multiUb chain on a substrate often signals it degradation, the authors propose that CDC34 may target itself for destruction. However the region of the CDC34 polypeptide containing the ubiquitinated lysine residues is not essential for its cell cycle function (Kolman *et al.*, 1992; Silver *et al.*, 1992) leading to the suggestion that overlapping mechanisms exist to inactivate CDC34, such as phosphorylation (Goebl *et al.*, 1994). UBC1 also forms a multiUb chain on itself *in vitro* in an intramolecular reaction (Hodgins *et al.*, 1996). This reaction may be advantageous because the attachment of a prefabricated multiUb chain would rapidly target proteins for destruction. Thus at least three E2s can be ubiquitinated but the functional significance of these modifications remains to be addressed.

Regions of E2 Specificity

It can be imagined that regions responsible for specificity may either map precisely to the same area on the surface of each E2, overlap with one another, or at the extreme, exist as distinct nonoverlapping regions (figure 1). Evidence exists to support the hypothesis that these regions map to similar positions in E2s. Residue 120, for example, was found in this study to be important for RAD6 DNA repair activity, and the corresponding residue in CDC34 (residue 139) is required for efficient CDC34 activity (Liu *et al.*, 1995). In addition proline 62 (UBC4 numbering), which is conserved in all E2s, has been shown to be necessary for the activity of several E2s. Alteration of this residue to a serine in RAD6, CDC34, UBC4, or UBC9 creates a temperature sensitive polypeptide (Ellison *et al.*, 1991; Arnason, 1995; Betting and Seufert, 1996). At least concerning RAD6, the substitution of the proline residue is not simply interfering with Ub transfer as only certain RAD6 functions are affected; substitution of the proline residue adversely affects the growth function of RAD6, but not its cell division or DNA repair functions (Ellison *et al.*, 1991). As suggested below, the proline residue is likely part of a determinant required for E2 specificity.

The proline residue referred to above is located in the conserved sequence $PX_1X_2(P/S/A)P$ present in all E2s (figure 2). In UBC4 X_1 and X_2 are F63 and K64 respectively. F63 is involved in UBC4 specificity as substitution of the corresponding residue in a functionally distinct E2 created a derivative that had significant UBC4 function (Chapter 3). As I have already discussed, K64 is necessary for ubiquitination of UBC4 (Gwozd *et al*, 1995); the involvement of F63 in UBC4 ubiquitination was not tested. Interestingly in UBC1, like UBC4, this sequence is PFKPP. UBC1 and UBC4/5 constitute a family of E2s that are necessary for cell growth and viability (Seufert *et al.*, 1990). Mutants that are deleted for individual genes are viable but mutants deleted for all three genes are not. Thus UBC1 and UBC4/5 possess overlapping functions. It is possible that part of their functional overlap is due to the PFKPP sequence which interacts with common substrates or trans acting factors. However the UBC1 and UBC4/5 catalytic domains possess a high degree of sequence identity so other regions are also likely to be involved in their common functions. UBC6 and UBC8 also have a similar $PX_1X_2(P/S/A)P$ motif to UBC4, where X_1 is a tyrosine and X_2 is a lysine residue. Tyrosine is often a conservative substitution for phenylalanine. UBC4 and UBC6 possess some common functions; they are both involved in the degradation of MATα2 (Chen *et al.*, 1993) and are high copy suppressors of the secretion mutant *sec61* (Jentsch, 1992; Sommer and Jentsch, 1993). Whether these functions are a result of common factors interacting at the PFKPP motif must be tested, nevertheless it is an attractive possibility. *ubc8* null mutants on the other hand have no detectable phenotype (Qin *et al.*, 1991). UBC8 is one of two E2s with a residue other than proline at the P/S/A position. This may change the functional determinant at this site, and thus it is not unexpected that UBC4 and UBC8 do not have functions in common. The $PX_1X_2(P/S/A)P$ sequences of other E2s are different and cannot be easily grouped accordingly (figure 2); this may indicate that, for these E2s, distinct trans acting factors bind to this region. The $PX_1X_2(P/S/A)P$ sequence is likely important for the functions of all E2s and should be investigated.

Although the above evidence supports the idea that E2s share common regions for specificity, there is also evidence that E2s use distinct regions. Residues 120 and 123 are important for the DNA repair activity of RAD6, but the corresponding residues in UBC4 are not involved in its function (Chapter 3). In addition when residues conserved in homologues of both UBC4 and RAD6, but different between the two families, were substituted, only four of the twenty residues were identified as being important to E2 function (Chapter 3). If RAD6 and UBC4 did use corresponding regions it would be expected that more residues would have been identified that are involved in their specificities. Furthermore, the four residues were only involved in the activities of either one or the other of the E2s, not both. Thus the emerging picture of E2 specificity is one in which E2s use both corresponding and distinct regions for specific interactions.

It is interesting that a significant number of the residues involved in E2 specificity, identified in this study and by other groups, are present on the same surface of the molecule as the catalytic cysteine (for example see figures 5 and 6, Chapter 3). It is unlikely a coincident that specificity determinants and Ub are present on the same surface of the molecule. This probably allows E2s to simultaneously identify their interacting partners, possibly E3s or substrates, and transfer Ub to them. Nevertheless, residues on the opposite face of the molecule have also been determined to be important for specific E2 functions (McDonough et al, 1995; Wing *et al.*, 1996).

A Region Involved in RAD6 Function

Combining residues identified in this study and by McDonough *et al.* (1995) reveals a region important to RAD6 function. Residues 120 and 123 (Chapter 3) and 71, 86, and 90 (McDonough *et al.*, 1995) are clustered around the active site cysteine. Further mutational analysis in this area may identify other residues important to RAD6 functions, therefore defining the boundaries of this region.

Although a significant number of mutants have been made in *RAD6*, several residues that are highly conserved in all RAD6 homologues have not yet been tested for their participation as functional determinants. This is clearly seen in figure 3 where the green residues indicate positions that have been substituted in RAD6 (Chapter 3; Watkins *et al.*, 1993; McDonough *et al.*, 1995). The red residues are highly conserved residues found in all RAD6 homologues that thus far have not been substituted, but are logical targets for mutagenesis.

Isolation of Proteins that Interact with UBC4 and RAD6

The work I have presented represents only a beginning towards understanding the structural basis of E2 function. The logical step in completing this understanding naturally involves the identification of E2 accessory proteins that participate in their functional discrimination. Ultimately E2 derivatives that are inactive with respect to specific functions could prove to be useful tools for biochemical and genetic screens to isolate E2 transacting factors involved in specificity or regulation. rad6Δ F65 N82 and rad6Δ D120 V123 exhibit considerable promise in this regard. First the substituted residues are found on the surface of the molecules, and therefore are probably involved in protein-protein interactions. Second both derivatives retain their native conformation as shown by the retention of RAD6 DNA repair activity by rad6Δ F65 N82, and the ability of rad6Δ D120 V123 to form a Ub thiolester conjugate.

The particular phenotypes of the E2 null mutants also enable these derivatives to be used in genetic screens. The *ubc4ubc5* null strain is temperature sensitive and is not viable when grown at 38°C (Seufert and Jentsch, 1990). Expression of rad6Δ F65 N82 partially restores the temperature resistance of the *ubc4ubc5* null strain but cells are still relatively heat sensitive; therefore this phenotype affords an excellent way of screening for proteins that interact with rad6 F65 N82 and ultimately UBC4. The *ubc4ubc5* null strain would be cotransformed with a plasmid expressing rad6Δ F65 N82 and a cDNA library plasmid. Overexpression of a protein that normally associates with rad6Δ F65 N82 is expected to stabilize the interaction and thereby increase the temperature resistance of the *ubc4ubc5Δ* cells. Colonies that can grow at an elevated temperature would be selected, and the library plasmid isolated. The effect would be tested for its dependence on the presence of both rad6Δ F65 N82 and the library plasmid. The gene sequence of the library plasmid insert would then be determined, and the encoded protein characterized.

Similarly, the *rad6* null strain is extremely sensitive to UV irradiation (Cox and Parry, 1968) and expression of rad6Δ D120 V123 does not relieve this sensitivity. Therefore this phenotype provides a way of isolating binding factors that interact with RAD6 at these positions. Presumably rad6Δ D120 V123 is inactive because substitution of residues 120 and 123 disrupts the association with a protein responsible for RAD6 DNA repair. If the interaction of the protein with rad6Δ D120 V123 is weak it may be possible to increase UV resistance through its overexpression. *rad6* null cells cotransformed with rad6Δ D120 V123 and a cDNA library plasmid would be tested for their ability to survive UV irradiation that would normally kill cells expressing the rad6Δ derivative alone. Library plasmids from cells that survive would be tested to ensure that the effect is dependent on the rad6Δ derivative. In addition, colonies would be scored for growth versus no growth which makes the screening process simple. Again the protein encoded by the library plasmid would be characterized.

Until proteins that interact with these E2s are identified, an understanding of the structural basis of ubiquitin target recognition will remain largely incomplete.

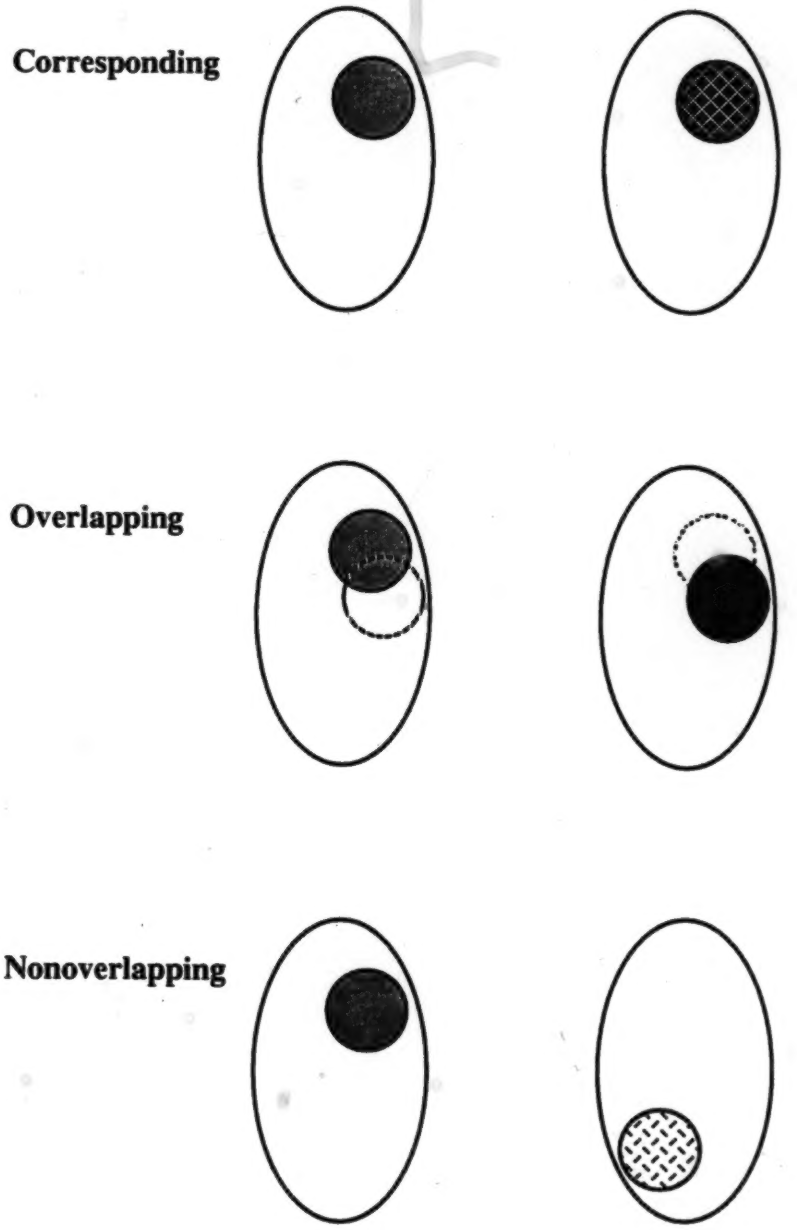


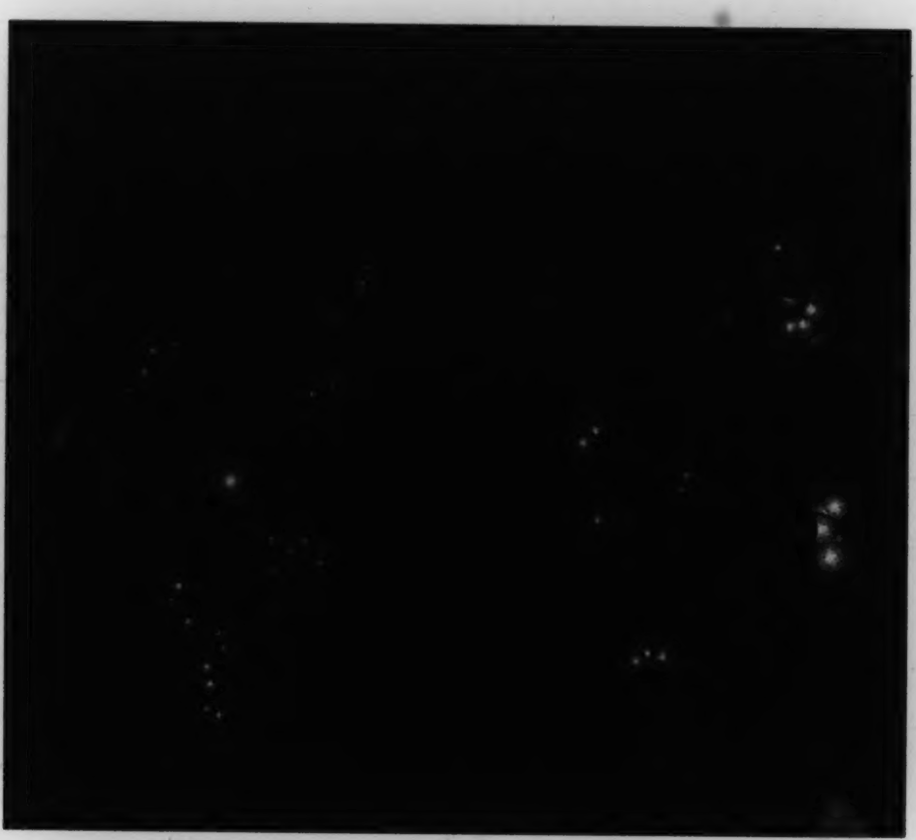
Figure 1: Potential positions of E2 specificity determinants. The ovals represent functionally distinct ubiquitin conjugating enzymes.

E2	Function	
UBC4/5	degradation of short-lived and abnormal proteins MATα2 degradation high copy suppressor of *sec61* secretion mutant	..P**FK**PP..
UBC1	overlapping functions with UBC4/5 in cell growth and viability required after sporulation/germination	..P**FK**PP..
UBC6	high copy suppressor of *sec61* secretion mutant MATα2 degradation	..P**YK**PP..
UBC8	unknown	..P**YKS**P..
UBC2/ RAD6	DNA repair	..P**NK**PP..
UBC3	cell cycle progression G1-S	..P**FS**PP..
UBC7	cadmium resistance MATα2 degradation	..P**LS**PP..
UBC9	cell cycle progression G2-M	..P**SK**PP..
UBC10	peroxisome biogenesis	..P**MN**PP..
UBC11	?	..P**FH**PP..
UBC12	?	..P**IE**PP..
UBC13	?	..P**MEA**P..

Figure 2: Comparison of $PX_1X_2(P/S/A)P$ protein sequences in *Saccharomyces cerevisiae* E2s.

Figure 3

Three dimensional space-filling images of the *A. thaliana* RAD6 homologue, rotated by 180°, showing the active site cysteine (yellow) and substituted RAD6 residues (green). Substitutions include those made in this study and in studies by Watkins *et al.* (1993) and McDonough *et al.* (1995). Residues highly conserved in all ubiquitin conjugating enzymes are colored blue. All other residues are colored from white to red; the darker the shade of red, the more highly conserved the residue is among the RAD6 homologues. The images were reconstructed from the crystallographic coordinates (Cook *et al.*, 1992) using the molecular modeling package Insight II (Biosym/MSI, San Diego).



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